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(54) Title: CYTOKININ RESPONSE REGULATORS AND USES THEREOF

(57) Abstract: The invention generally features methods for increasing yield, shoot formation, and delaying senescence in plants with the use of transgenes that regulate the cytokinin response. The invention also features plants and plant components that harbor the transgene(s).

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CYTOKININ RESPONSE REGULATORS AND USES THEREOF

Background of the Invention

This invention relates to genetically-engineered plants having increased yield and productivity, shoot, leaf and meristem formation, enhanced photosynthesis, and delayed senescence.

Despite long recognition of cytokinins as essential plant hormones involved in diverse processes of plant growth and development, including cell division, shoot initiation, leaf and root differentiation, chloroplast biogenesis, apical dominance, and senescence, the molecular and biochemical mechanisms underlying cytokinin actions have not been elucidated (Davies, *Plant Hormones: Physiology, Biochemistry and Molecular Biology*,1995, Kluwer Academic Publishers, Dordrecht; Meijer and Murray, *Curr. Opin. Plant Biol.* 4: 44-9, 2001; Mok and Mok, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 89-118, 2001; Quirino et al., *Trends Plant Sci.* 5: 278-82, 2000). Recent genetic identification of *Arabidopsis* hybrid histidine protein kinases (AHKs), CKI1 and CRE1, in cytokinin signalling (Kakimoto, *Science* 274: 982-5, 1996; Suzuki et al., *Plant Cell. Physiol.* 42: 107-13, 2001; Inoue et al., *Nature* 409: 1060-3, 2001), the characterization of *Arabidopsis* response regulators (ARRs) as cytokinin primary response genes (D'Agostino et al., *Plant Physiol.* 124: 1706-17, 2000; Kiba et al., *Plant Cell Physiol.* 40: 767-71, 1999), and the existence of *Arabidopsis* histidine-containing phosphotransmitters (AHPs; Suzuki, Imamura, et al., *Plant Cell Physiol.* 39: 1258-68, 1998), have implicated the involvement of a two-component phosphorelay mechanism in cytokinin signal transduction. However, cellular and molecular evidence is lacking in supporting a complete cytokinin signalling circuit in plant cells.

Two-component circuitry, consisting of a histidine kinase (HK) sensor and a response regulator (RR) output, are responsible for signal transduction in most prokaryotic and some eukaryotic systems. The signalling pathway is initiated by

a HK sensor and mediated by phosphotransfer between a conserved histidine (His) residue in HKs or histidine-containing phosphotransmitters (HPs) and a conserved aspartate (Asp) residue in RRs (Wurgler-Murphy and Saito, *Trends Biochem. Sci.* 22: 172-6, 1997; Stock et al., *Annu. Rev. Biochem.* 69: 183-215, 2000). Since there are only one HK, one HP, and two RRs in *S. cerevisiae*, it was previously believed that two-component signal transduction has limited function in eukaryotes (Stock et al., *supra*). However, the completion of the *Arabidopsis* genome has revealed over 40 genes encoding putative two-component signal transducers, AHKs, AHPs and ARRs, suggesting a significant involvement of the ancient and conserved signalling mechanism in many facets of plant cell regulation (Urao, et al., *Trends Plant Sci.* 5: 67-74, 2000). The identification of conserved HK signature motifs and/or activity in the photoreceptor phytochrome, putative osmosensor, and the ethylene and cytokinin receptors in *Arabidopsis* further supports this view (Inoue et al., *supra*; Urao, et al., *supra*; Yeh and Lagarias, *Proc. Natl. Acad. Sci. U SA* 95: 13976-81, 1998; Bleecker and Kende, *Annu. Rev. Cell Dev. Biol.* 16: 1-18, 2000).

In eukaryotic signal transduction, the two-component circuit often provides a link between the HK sensor to a MAP kinase (MAPK) signaling cascade. For example, the osmosensing signal transduction pathway in yeast is mediated by the SLN1/YPD1/SSK1 phosphorelay. The RR SSK1 then activates the HOG1 MAPK cascade in the cytosol to control gene expression (Maeda et al., *Nature* 369: 242-5, 1994; Posas et al., *Cell* 86: 865-75, 1996; Posas and Saito, *EMBO J.* 17: 1385-94, 1998). It has been speculated that plant HKs such as the ethylene receptor (ETR1) and a putative osmosensor (AHK1) can initiate a phosphorelay and transmit the signal through a MAPK cascade (Bleecker and Kende, *supra*; Gamble et al., *Proc. Natl. Acad. Sci. U SA* 95: 7825-9, 1998: Clark et al., *Proc. Natl. Acad. Sci. U SA* 95: 5401-6, 1998; Urao et al., *Plant Cell* 11: 1743-54, 1999). Due to the lack of physiological plant cell assays, the mechanisms of HK action and the signal transduction pathways of any HK-mediated plant responses, including cytokinin signalling, remain obscure.

Summary of the Invention

The present invention capitalizes on the discovery that manipulation of the expression of cytokinin response regulators increases plant yield growth, and productivity.

In one aspect, the invention features a method for increasing yield in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a B-type response regulator operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein the B-type response regulator is expressed in the cells of the transgenic plant, thereby increasing yield in the plant. In preferred embodiments, the B-type response regulator is a crucifer B-type response regulator (for example, ARR1 (SEQ ID NO.: 2); ARR2 (SEQ ID NO.: 3); and ARR10 (SEQ ID NO.: 9)).

In a second aspect, the invention features a method for increasing yield in a plant, the method including the steps of: (a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein expression of the transgene reduces expression of an A-type response regulator in the cells of the plant, thereby increasing yield in the plant. In preferred embodiments, the A-type response regulator is a crucifer A-type response regulator (for example, ARR 4 (SEQ ID NO.: 5); ARR 5 (SEQ ID NO.: 6); ARR 6 (SEQ ID NO.: 7); and ARR 7 (SEQ ID NO.: 8)). In other preferred embodiments, the transgene expresses an antisense molecule of A-type response regulator; a dominant negative gene product of A-type response regulator; or expression of the transgene results in the co-suppression of the A-type response regulator.

In a third aspect, the invention features a method for increasing shoot formation in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a B-type response regulator operably

linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein the B-type response regulator is expressed in the cells of the plant, thereby increasing shoot formation in the plant. In preferred embodiments, the method includes the use of a B-type response regulator that is a crucifer B-type response regulator.

In a fourth aspect, the invention features a method for increasing shoot formation in a plant, the method including the steps of: (a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein expression of the transgene reduces expression of an A-type response regulator in the cells of the plant, thereby increasing shoot formation in the plant.

In a fifth aspect, the invention features a method for delaying senescence in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a B-type response regulator operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein the B-type response regulator is expressed in the cells of the plant, thereby delaying senescence in the plant.

In a sixth aspect, a method for delaying senescence in a plant, the method including the steps of: (a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein expression of the transgene reduces expression of an A-type response regulator in the cells of the plant, thereby delaying senescence in the plant.

In a seventh aspect, the invention features a method for increasing yield in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein histidine kinase is

expressed in the cells of the plant, thereby increasing yield in the plant. In preferred embodiments, the histidine kinase is a crucifer histidine kinase (for example, CKI1 (SEQ ID NO.: 13) and CRE1 (SEQ ID NO.: 18)).

In an eighth aspect, the invention features a method for increasing shoot formation in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein histidine kinase is expressed in the cells of the plant, thereby increasing shoot formation in the plant.

In a ninth aspect, a method for delaying senescence in a plant, the method including the steps of: (a) introducing into plant cells a transgene including DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and (b) regenerating a plant from the transformed cells, wherein histidine kinase is expressed in the cells of the plant, thereby delaying senescence in the plant.

The invention further includes plants and plant components expressing one, or a combination of two or more, of the aforementioned transgenes. The invention also encompasses methods of generating these plants or plant components, which may involve introducing the transgenes individually to separate plants or plant components, and then crossing the appropriate genotypes to get the desired transgene combination. Alternatively, one or more constructs expressing a desired combination of transgenes may be generated and then introduced into the plant or plant component.

For example, the invention also relates to a plant or plant component comprising at least one transgene encoding (i) an A-type response regulator polypeptide, (ii) an antisense A-type response regulator RNA, (iii) a dominant negative A-type response regulator polypeptide, (iv) a B-type response regulator polypeptide, (v) an antisense B-type response regulator RNA, (vi) a dominant negative B-type response regulator, (vii) an antisense HK RNA, (viii) a dominant

negative HK polypeptide, or any combination of (i) –(viii). In addition, the invention includes any of the aforementioned plants further including a transgene encoding a histidine kinase polypeptide.

The invention further includes a plant or plant component that includes: (a) a first transgene encoding (i) an A-type response regulator polypeptide, (ii) an antisense A-type response regulator RNA, or (iii) a dominant negative A-type response regulator polypeptide or combination thereof; (b) a second transgene encoding (iv) a B-type response regulator polypeptide, (v) an antisense B-type response regulator RNA, (vi) a dominant negative B-type response regulator or combination thereof, and (c) a third transgene encoding (vii) a HK polypeptide; (viii) an antisense HK RNA, (ix) a dominant negative HK polypeptide or combination thereof.

Exemplary plants which are useful in the methods of the invention, as well as for generating the plants (or plant cells, plant components, plant tissues, or plant organs) of the invention, include dicots and monocots, such as sugar cane, wheat, rice, maize, sugar beet, barley, manioc, crucifer, mustard, potato, soybean, sorghum, cassava, banana, grape, oats, tomato, millet, coconut, orange, rye, cabbage, apple, eggplant, watermelon, canola, cotton, carrot, garlic, onion, pepper, strawberry, yam, papaya, peanut, onion, legume, bean, pea, mango, and sunflower.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-plant component expression control permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein, includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "plant component" is meant a part, segment, or organ obtained from an intact plant or plant cell. Exemplary plant components include, without limitation, somatic embryos, leaves, stems, roots, flowers, tendrils, fruits, scions, and rootstocks.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a nucleic acid sequence (e.g., a recombinant DNA sequence) which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By "yield" or "plant yield" is meant increased growth (e.g., crop growth) or increased biomass. For example, increased yield results from increased shoot growth or meristem formation. Plants expressing the genes disclosed herein exhibiting increased yield can be selected by visual observation, for example by comparison with a wild-type plant.

By "reducing expression" or "reduces expression" is meant a decrease in the level of gene expression (for example, expression of a gene encoding a A-type response regulator) by at least 30-50%, preferably by at least 50-80%, and more preferably by at least 80-95% or greater relative to the level in a control plant (for example, a wild-type plant). Reduction of such expression levels may be accomplished by employing standard methods which are known in the art including, without limitation, antisense and co-suppression technologies, expression of a dominant negative gene product, or through the generation of mutated genes using standard mutagenesis techniques. Levels of negative

regulator polypeptide or transcript are monitored according to any standard technique including, but not limited to, northern blotting, RNase protection, or immunoblotting.

By "crucifer" is meant any plant that is classified within the Cruciferae family. The Cruciferae include many agricultural crops, including, without limitation, rape (for example, *Brassica campestris* and *Brassica napus*), broccoli, cabbage, brussel sprouts, radish, kale, Chinese kale, kohlrabi, cauliflower, turnip, rutabaga, mustard, horseradish, and *Arabidopsis*.

By "a promoter functional in a plant cell" is meant any minimal sequence sufficient to direct transcription in a plant cell. Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external or internal agents (for example, cytokinins), or elements that are capable of cycling gene transcription; such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

The invention provides a number of important advances and advantages for improving and enhancing agronomically important traits such as photosynthesis, productivity, yield, leaf, shoot, and meristem formation, and delaying senescence. In particular, the invention provides for increased production efficiency, as well as for improvements in quality and yield of crop plants and ornamentals. Thus, the invention contributes to the production of high quality and high yield agricultural products; for example, fruits, ornamentals, vegetables, cereals, and field crops. Genetically-improved seeds and other plant products that are produced using plants expressing the genes and methods described herein also render farming possible in areas previously unsuitable for agricultural production The mechanisms disclosed herein for increasing plant yield and productivity is expected to be ubiquitous throughout the plant kingdom.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Figure 1A is a bar graph showing the specificity of plant hormone responses in the *Arabidopsis* mesophyll protoplast transient expression system.

Figure 1B is a graph depicting a cytokinin dose response for the *ARR6* promoter induction.

Figure 1C is a bar graph showing the induction of the *ARR6* promoter by different cytokinins.

Figure 1D shows that CKI1 activation of *ARR6-LUC* requires HK activity and phosphorelay. The photograph (top) shows an autoradiogram depicting the expression of CKI1 proteins. The bar graph (bottom) shows the results of transfected protoplasts that were incubated for 3 hours to allow effector gene expression before cytokinin treatment for another 3 hours.

Figure 1E shows that CRE1 confers cytokinin hypersensitivity on the activation of the *ARR6-LUC* reporter. The photograph (top) shows an autoradiogram depicting the expression of *Arabidopsis* HK proteins. The results of this incubation with cytokinin are shown in the bar graph (bottom).

Figure 1F shows a series of a photomicrographs showing that CKI1 is localized at the plasma membrane.

Figure 2A demonstrates that AHP acts as a shuttle between the cytosol and nucleus in cytokinin signalling. The photograph (top) depicts an autoradiogram demonstrating expression of AHP proteins. The bar graph (bottom) shows the effect of cytokinin treatment on plasmid expression.

Figure 2B shows a series of photomicrographs demonstrating that cytokinin induces transient AHP translocation.

Figure 3A shows negative and positive regulation by ARRs. The photograph depicts an autoradiogram (top) showing the expression of *Arabidopsis* ARR proteins. The bar graph (bottom) shows the activity of *ARR6* promoter activity when cells were then incubated with or without 100 nM *t*-zeatin for another 3 hours before the activity was measured.

Figure 3B shows that ARRs act downstream of CKI1 in cytokinin signalling.

Figure 3C shows a series of photomicrographs demonstrating that *Arabidopsis* ARRs are localized in the nucleus.

Figure 4A is a photograph showing that ectopic expression of ARR2 is sufficient to promote cytokinin responses in transgenic tissues and plants when exposed to auxin.

Figure 4B is a photograph showing that ectopic expression of ARR2 is sufficient to promote cytokinin responses in transgenic tissues and plants when exposed to both auxin and cytokinin.

Figure 4C is a photograph demonstrating that cytokinin signaling delays dark-induced senescence in transgenic *Arabidopsis* plants.

Figure 5 is a schematic of a model of the cytokinin signal transduction pathway in *Arabidopsis*.

Detailed Description

Overview

Cytokinins are essential plant hormones involved in shoot meristem and leaf formation, cell division, chloroplast biogenesis, and senescence. Although hybrid histidine protein kinases (HKs) have been implicated in cytokinin perception in *Arabidopsis*, the action of HK receptors and downstream signalling pathways remain elusive in plant cells. To study the action of HK receptors and downstream signaling pathways, a mesophyll protoplast transient assay based on the transcription activation of a cytokinin primary response gene *ARR6* in *Arabidopsis* was developed. The use of this high-throughput cell assay for functional genomic analysis of the two-component regulators has provided a powerful tool to decipher the first complete cytokinin signalling pathway. Rather than following the established eukaryotic HK and MAPK cascade paradigm,

plant two-component signalling can integrate multiple HK activities to common AHPs as cytoplasm and nuclear shuttles, and distinct nuclear ARRs as master regulatory outputs.

As is described in more detail below, a new eukaryotic two-component signalling circuit that initiates cytokinin signalling by employing distinct hybrid HK activities at the plasma membrane has been identified. In particular, AHPs are shown to act as novel eukaryotic signalling shuttles between the cytoplasm and nucleus in a transient and cytokinin-dependent manner. The short signalling circuit reaches the nuclear target genes by de-repressing nuclear response regulators ARR1, ARR2, and ARR10 as transcription activators. The cytokinin-inducible ARR4, ARR5, ARR6, and ARR7 act as transcription repressors, suggesting a negative feedback loop. Thus, the *Arabidopsis* cytokinin signal transduction pathway consists of four major steps: HK sensing and signalling, AHP translocation, ARR de-repression, and a negative feedback loop by cytokinin-inducible ARRs. Transgenic tissue and plant analyses support the importance of this core signalling pathway in diverse cytokinin responses. Furthermore, ectopic expression of the master regulator ARR2 as the rate-limiting two-component output in transgenic *Arabidopsis* was sufficient to mimic cytokinin in promoting shoot meristem proliferation, leaf differentiation, expansion, and longevity.

The examples provided below are for the purposes of illustrating the invention, and should not be construed as limiting.

<u>Cytokinin Signal is Perceived and Initiated by the Multiple HK receptors</u>

To elucidate the regulatory circuitry in cytokinin signal transduction, a leaf cell assay based on cytokinin inducible transcription in *Arabidopsis* mesophyll protoplasts was developed. The 2.4 kb promoter of an *Arabidopsis* cytokinin primary response gene (SEQ ID NO.: 1), encoding the response regulator 6 (*ARR6*), was fused to the firefly luciferase (LUC) reporter. In Fig. 1A, transfected protoplasts were incubated with or without 100 nM *t*-zeatin, 1 μM

indole-3-acetic acid (IAA), or 100 μM ABA. After 3 hours of incubation, LUC and GUS activities were determined. In transfected protoplasts, the activity of *ARR6-LUC* was specifically induced by cytokinin but not by other plant hormones such as abscisic acid (ABA) or auxin (Fig. 1A).

In the same system, the *GH3* promoter was only activated by auxin, whereas the *RD29A* promoter was solely induced by abscisic acid (ABA), demonstrating the specificity of three plant hormone responses in *Arabidopsis* mesophyll protoplasts (Fig. 1A). The activity of *ARR6-LUC* was induced by physiological concentration of a natural cytokinin *trans*-zeatin (*t*-zeatin) from 1 to 100 nM (Fig. 1B).

To further show the specificity of the cytokinin response, various active and inactive cytokinin analogues were examined. Only the active cytokinins, *t*-zeatin, 2-isopentenyladenine (2-IP; 100 nM) and benzyladenine (BA; 100 nM), induced the reporter gene *ARR6-LUC* (Fig. 1C).

The response in the protoplast system is similar to the cytokinin activation of various *ARR* genes shown *in planta* (D'Agostino et al., *supra*; Kiba et al., *supra*). Thus, we have established a reliable and physiological system to dissect the regulatory components in the cytokinin signal transduction pathway in *Arabidopsis*.

Cytokinin Signalling by Distinct HK Receptors and Phosphorelay

Despite the genetic identification of histidine kinases (HKs) as receptors for the plant hormones ethylene and cytokinin in *Arabidopsis*, whether or where HK activity is important for signal transduction in plant cells remains an unresolved question due to the lack of physiological plant cell assays. CKI1, a hybrid HK with a conserved response regulator (RR) receiver domain has been implicated in cytokinin responses (Kakimoto, *supra*). The enhanced CKI1 expression in *Arabidopsis shooty callus* mutants also supports its role in cytokinin signalling (Frank et al., *Plant Physiol.* 122: 721-9, 2000). However, the biochemical mechanism of CKI1 function and its role in cytokinin signalling are

still unknown. To determine whether CKI1 mediates cytokinin responsive transcription, the full-length *CKI1* gene (SEQ ID NO.: 13) was tagged with double haemagglutinin epitope (DHA) sequence and fused to a constitutive promoter, and co-transfected with *ARR6-LUC* into the *Arabidopsis* protoplasts. Specifically, protoplasts were cotransfected with the *ARR6-LUC* reporter and the effector plasmid expressing CKI1 (SEQ ID NO.: 13), CKI1(H405Q) (SEQ ID NO.: 14), CKI1(D1050N) (SEQ ID NO.: 15), CKI1-GFP, or mER7-GFP (control). As shown in Fig. 1D, the wild-type CKI1 activated the *ARR6* promoter without exogenous *t*-zeatin. It is possible that over-expression of CKI1 renders protoplasts hypersensitive to endogenous cytokinin and/or exceeds the capacity of negative regulators. Alternatively, CKI1 could encode a constitutively active HK connected to the cytokinin signal transduction pathway. Cytokinin treatment hardly enhanced the reporter gene activity in the presence of CKI1 (Fig. 1D).

To determine whether the HK activity and phosphorelay are required for CKI1 activation of the *ARR6* promoter, the conserved His 405 and Asp 1040 residues in the HK and phosphorylation sites, respectively, were mutated. Specifically, protoplasts were transfected with plasmid DNA expressing CKI1 (SEQ ID NO.: 13), CKI (H405Q) (SEQ ID NO.: 14) and CKI1(D1040N) (SEQ ID NO.: 15), or vector DNA as a control. The [^{35}S]methionine labeled protein from transfected protoplasts was immunoprecipitated with anti-HA and proteinA-agarose, and analyzed by SDS-PAGE. Despite comparable expression of the CKI1(H405Q) and CKI1(D1050N) mutants as the wild-type CKI1, neither could activate the expression of *ARR6-LUC* (Fig. 1D). Furthermore, the CKI1(H405Q) mutant served as a strong dominant negative mutant to block the activation of the *ARR6* promoter by exogenous *t*-zeatin. The CKI1(D1040N) mutant is less potent. These results indicated that HK activity and phosphorelay from His 405 to D1040 are required for the CKI1 function in activating cytokinin signalling. Moreover, the dominant negative CKI1(H405Q) mutant might interfere with cytokinin perception and/or disturb downstream signalling.

Recently, another *Arabidopsis* hybrid HK, CRE1/AHK4/WOL, has been shown to be a cytokinin receptor. The evidence is based on the inability of the *cre1* mutant to respond to cytokinin in the shoot induction assay and its ability to complement HK mutants of budding and fission yeast and *E. coli* in a cytokinin-dependent manner (Suzuki et al., *supra*; Inoue et al., *supra*; Mahonen et al., *Genes Dev.* 14: 2938-43, 2000). Since *CRE1/AHK4/WOL* is predominantly expressed in roots (Mahonen et al., *supra*; Ueguchi et al., *Plant Cell Physiol.* 42: 231-5, 2001) and the *cre1* and *wol* recessive mutants lack an obvious leaf phenotype (Inoue et al., *supra*; Mahonen et al., *supra*; Ueguchi et al., *supra*), it is possible that other closely related *Arabidopsis* HK genes, such as *AHK2* and *AHK3*, and *CKI1* provide cytokinin receptor functions in promoting shoot meristem and leaf formation, and delaying leaf senescence (Inoue et al., *supra*; Ueguchi et al., *supra*).

To directly test the function of these HKs in cytokinin signalling, constructs expressing AHK2 (SEQ ID NO.: 16), AHK3 (SEQ ID NO.: 17), or CRE1(AHK4/WOL) (SEQ ID NO.: 18) were cotransfected into *Arabidopsis* protoplast with the cytokinin-inducible reporter *ARR6-LUC*. Specifically, protoplasts were cotransfected with the *ARR6-LUC* reporter and the effector plasmid expressing AHK2, AHK3, CRE1(AHK4/WOL), CRE1(H467Q) (SEQ ID NO.: 19), CRE1(D973N) (SEQ ID NO.: 20), or mER7-GFP (control), and were analyzed after 6 hours of cytokinin treatment. Different from the CKI1 activation, over-expression of AHK2, AHK3, or CRE1(AHK4/WOL) did not enhance the expression of *ARR6-LUC* from the basal level in the absence of exogenous cytokinin. However, cytokinin treatment resulted in further activation of the reporter, especially for CRE1(AHK4/WOL) (Fig.1E). The requirement of the conserved His 467 and Asp 973 residues in the HK and the receiver domain, respectively, for the CRE1 activity was also tested. In Fig. 1E top, the expression of *Arabidopsis* HK proteins was detected by [^{35}S]methionine-labeled AHK2 (SEQ ID NO.: 26), AHK3 (SEQ ID NO.: 27), CRE1(AHK4/WOL) (SEQ ID NO.: 28), CRE1(H467Q) (SEQ ID NO.: 29), and CRE1(D973N) (SEQ ID NO.:

30) that were immunoprecipitated and analyzed by SDS-PAGE. CRE1(H467Q) and CRE1(D973N) mutants lost their ability to further enhance *ARR6-LUC* expression in the presence of exogenous cytokinin (Fig. 1E).

Moreover, both mutants also imposed a dominant negative effect on blocking cytokinin signalling. Similar to the CKI1 mutants, the His mutant is more potent than the Asp mutant, perhaps due to the flexible phosphorelay process between mutant and wild-type HKs. These results indicated that cytokinin signals are sensed by multiple HK receptors with different or overlapping expression patterns. Although CRE1 is predominantly expressed in roots (Mahonen et al., *supra*; Ueguchi et al., *supra*), it can certainly function in leaf cells as a cytokinin receptor. However, CKI1 and CRE1 represent two different types of cytokinin receptors that require HK activity and phosphorelay to initiate cytokinin signalling, but have different regulatory mechanisms and probably distinct affinity or specificity for cytokinins.

To gain insight into where the cytokinin signalling is perceived, the sub-cellular localization of CKI1 was examined using the CKI1-GFP fusion. We first confirmed that CKI1-GFP acted similarly as CKI1-HA in the protoplast assay based on *ARR6-LUC* activation (Fig. 1D). Confocal microscopy showed that CKI1 is mainly localized to the plasma membrane but not in the cytosol, ER or nucleus, indicated by various GFP markers (Fig. 1F). Specifically, protoplasts were transfected with CKI1-GFP or various GFP marker plasmid DNA. Tunicamycin (1 μg/ml) treatment was for 12 hours. The transfected protoplasts were analyzed with a confocal microscope. The subcellular GFP markers are "CAT-GFP" for the cytosol, "ER-GFP" for the endoplasmic reticulum, and "N-GFP" (HSP-GFP) for the nucleus. Since the N-terminus of CKI1 is replete with putative glycosylation sites, the effect of a glycosylation inhibitor, tunicamycin, in CKI1-GFP expression, localization or function was examined. Although tunicamycin did not affect the expression of other GFP constructs, the expression

of CKI1-GFP was completely abolished (Fig. 1F). This result showed that the glycosylation of CKI1 is important for its stability, processing and/or trafficking in plant cells.

HPs Acts as a Shuttle Between the Cytosol and Nucleus Shuttles in Cytokinin Signalling

The studies of multistep phosphorelay in yeast osmosensing and in various prokaryotic systems have shown the use of histidine-containing phosphotransmitters (HP) as signal transducers between receptors and response regulators (Stock et al., *supra*; Posas et al., *supra*; Uhl and Miller, *EMBO J.* 15: 1028-36, 1996). The analysis of the *Arabidopsis* genome has revealed at least five genes encoding putative AHPs with unknown physiological functions. To determine whether AHPs are involved in the cytokinin signal transduction, AHP1, AHP2, and AHP5 were cloned into the plant expression vector and tested in the protoplast cytokinin response assay based on the *ARR6-LUC* reporter activity. AHP1, AHP2, and AHP5 slightly increased the expression of the *ARR6-LUC* with or without *t*-zeatin (Fig. 2A). Specifically, protoplasts were cotransfected with the *ARR6-LUC* reporter and an effector plasmid carrying AHP1 (SEQ ID NO.: 21), AHP2 (SEQ ID NO.: 22), AHP5 (SEQ ID NO.: 23), or AHP1 mutants (SEQ ID NOS.: 24 and 25). Transfected protoplasts were incubated for 6 hours to allow effector expression before cytokinin treatment for 3 hours. The AHP1 mutant, where the conserved His79 was replaced with Gln, did not repress the cytokinin activation of *ARR6-LUC*. Even the AHP1(H77Q, H79Q; SEQ ID NO.: 25) double mutant was not able to inhibit the *ARR6-LUC* activation by cytokinin. AHPs and AHP1 mutants were expressed at comparable levels in the protoplast system (Fig. 2A top). This result indicated that AHPs are not the limiting factor or the key switch/regulators in cytokinin signalling. Their action as potential mediators may require cytokinin-dependent modification.

Previous *in vitro* studies have suggested that AHP1 and AHP2 could interact with the receiver domain of CKI1 (Nakamura et al., *Biosci. Biotechnol. Biochem.* 63: 1627-30, 1999).

To gain further *in vivo* support for the involvement of AHPs in cytokinin signalling, the action of AHP1-GFP in transfected protoplast in the absence or presence of cytokinin was followed using fluorescence microscopy. Specifically, protoplasts were transfected with *AHP1-GFP*, *AHP2-GFP*, or *AHP5-GFP* and incubated for 3 hours to allow sufficient expression. After *t*-zeatin (100 nM) treatment for 30 minutes, transfected cells were observed under a fluorescence microscope. As shown in Fig. 2B, AHP1-GFP was mainly localized in the cytoplasm without cytokinin stimulation, but was translocated into the nucleus by *t*-zeatin treatment. The cytokinin-dependent translocation of AHP1-GFP was transient, occurring within 30 to 90 minutes after cytokinin treatment (Fig. 2B). AHP2-GFP showed similar cytokinin-dependent translocation but not AHP5-GFP, suggesting their different functions in plant cells. Thus, AHP1 and AHP2 are believed to serve as shuttles and phosphorelay carriers between the cytokinin receptors and the downstream nuclear responses.

Opposite Regulation of Early Cytokinin Response Gene Transcription by Two Types of Nuclear RRs in Cytokinin Signalling

The requirement of HK activity and phosphorelay in the initiation of cytokinin signalling from CKI1 and CRE1, the cytokinin-inducible *ARR6* expression, the cytokinin-dependent translocation of AHPs into the nucleus, and the physical interaction of AHPs and ARRs in yeast (Suzuki et al., *Plant Cell Physiol.* 42: 37-45, 2001) suggest that some ARRs may act downstream in cytokinin signal transduction. There are two major subfamilies of *RR* genes in the *Arabidopsis* genome: the cytokinin-inducible A type and the DNA-binding B type (Urao, et al., *supra*; D'Agostino and Kieber, *Trends Biochem. Sci.* 24: 452-6, 1999; Sakai et al., *Plant J.* 24: 703-11, 2000). To investigate whether any *Arabidopsis* RRs are involved in cytokinin signalling, four representative A-type

ARR genes and three B-type *ARR* genes were cloned into a standard plant expression vector with an HA or GFP tag. Specifically, protoplasts were cotransfected with the *ARR6-LUC* reporter and the effector plasmid carrying *ARR1, ARR2, ARR4, ARR5, ARR6, ARR7, ARR10*, or various mutants, *ARR2* (D80N) (SEQ ID NO.: 10), *ARR4*(D95N) (SEQ ID NO.: 11), and *ARR6*(D76N) (SEQ ID NO.: 12). As shown in Fig. 3A, A-type ARRs, such as ARR4, ARR5, ARR6, and ARR7, repressed *ARR6-LUC* activity induced by 100 nM *t*-zeatin. Although ARR6 seemed to be expressed at lower abundance, its repression activity was the strongest.

In contrast, the B-type ARRs, such as ARR1, ARR2, and ARR10, dramatically activated *ARR6-LUC* expression. Over-expression of ARR1, ARR2, and ARR10 was sufficient to activate cytokinin signalling at different levels in the absence of exogenous cytokinin. ARR1 and ARR2 activated *ARR6-LUC* about 40- and 400-fold, respectively. Cytokinin treatment further enhanced the effect of ARR2 on *ARR6-LUC* to over 1000-fold. The lower activation by ARR1 could be due to its lower expression level in transfected protoplasts. In the absence of cytokinin, ARR10 could activate *ARR6-LUC* about 10 fold. Cytokinin enhanced the effect of ARR10 on the *ARR6-LUC* activity another 10-20 fold. The differential effect of ARR2 and ARR10 in activating cytokinin signalling could be attributed to differences in their intrinsic activities in DNA binding and/or transcription activation. It could also be due to their distinct affinity to endogenous repressors that likely exist in leaf protoplasts to prevent cytokinin signalling under unstimulated condition. Surprisingly, mutations of the conserved Asp residue in the receiver domains of ARR4(D95N), ARR6(D76N), and ARR2(D80N) did not lead to alteration of their repression and activation functions, respectively, in cytokinin signalling (Fig. 3A). The transfected cells were incubated for 3 hours to allow effector protein expression. This result is consistent with the model that phosphorelay stimulated by cytokinin may be involved in de-repression of the positive regulators like ARR1, ARR2, and

ARR10. Over-expression of these ARRs likely bypasses the negative regulation and causes constitutive cytokinin responses without the signal.

To further support the role of ARR2 in cytokinin signalling, we designed a dominant mutant of ARR2 (ΔARR2) (SEQ ID NO.: 4) containing only the DNA binding domain but not the putative transcription activation and the receiver domains (Sakai et al., *supra*). If ΔARR2 can compete with the endogenous ARRs in binding to the *ARR6* promoter without transcription activation, it may block cytokinin- or CKI1-dependent activation of *ARR6-LUC*. In Fig. 3B, protoplasts were cotransfected with the *ARR6-LUC* reporter alone or with the effector plasmids as indicated. Transfected protoplasts were incubated for 6 hours to allow effector protein expression before treatment with 100 nM *t*-zeatin for 3 hours. As shown in Fig. 3B, the dominant negative mutant of ARR2 could effectively block *ARR6-LUC* expression in the presence of 100 nM *t*-zeatin or CKI1, suggesting that ARR2 and/or ARR2-like proteins act downstream of cytokinin and CKI1-initiated phosphorelay as the nuclear targets in the cytokinin signal transduction pathway. Furthermore, the dominant negative effect of the CKI1(H450Q) mutant on the protoplast response to exogenous cytokinin could be bypassed by over-expression of the wild-type ARR2 (Fig. 3B). These epistasis analyses place ARR2 downstream of multiple cytokinin HK receptors. The finding of multiple ARR2 binding motifs, (G/A)GAT(T/C), in the promoter regions of *ARR6* and other cytokinin-inducible genes suggests that ARR2 could be a master regulator in cytokinin signalling (*Id.*). Exogenous cytokinin-initiated phosphorelay through CRE1, or AHK2 and AHK3 likely converges with the CKI1 phosphorelay on the common AHP shuttles and the nuclear ARR transcription activators.

To provide more evidence that the A-type and B-type ARRs are transcription repressors and activators, respectively, in cytokinin signalling, the sub-cellular localization of ARR6-GFP and ARR2-GFP was examined. In Fig. 3C, Protoplasts were transfected with *ARR2-GFP, ARR2(D80N)-GFP, ΔARR2-*GFP, or *ARR6-GFP* plasmid DNA and observed after 3-6 hours of incubation

with a fluorescence microscope. Both types of ARRs are exclusively localized in the nucleus regardless of the cytokinin treatment (Fig. 3C). There nuclear localization is likely independent of the phosphorylation state because ARR2(D80N)-GFP was found in the nucleus. The ARR2 dominant negative mutant (ΔARR2) was also localized in the nucleus (Fig. 3C). Thus, the cytokinin-dependent phosphorelay does not play a role in the nuclear localization and DNA-binding of ARRs or their intrinsic transcription activation or repression activities. The results support the model that cytokinin-initiated phosphorelay plays a role in releasing the sequestered activation-type ARR transcription factors from the yet unknown repressors (Fig. 5).

As depicted in Fig. 5, cytokinin signal is perceived internally or externally by multiple HKs at the plasma membrane (AHK2, AHK3, CKI1, or CRE1). Upon perception of the cytokinin signal, HKs initiate a signalling cascade via the phosphorelay that in turn results in the nuclear translocation of AHPs from the cytosol. Activated AHPs interact with sequestered ARRs or ARR complex and release the activation type of ARRs from putative repressor in the nucleus. The de-repressed ARRs bind to multiple *cis* elements in the promoter of target genes. The transcription activation is essential for cell proliferation, shoot formation, and delayed senescence. The activation of the repressor-type of *ARRs* as early cytokinin response genes provides a negative feedback mechanism that allows plant cells for fine-tuning or resetting the physiological state in diverse differentiation processes during plant growth and development (RD, response domain; BD, DNA binding domain; AD, transcription activation domain; PM, plasma membrane; N, nucleus; R, repressor).

This model is consistent with the findings that the N-terminus of ARR1 or ARR2 inhibits transcription based on a synthetic promoter (*Id.*), and the addition of cytokinin further enhances the effects of ARR1, ARR2 and ARR10 over-expression on the *ARR6-LUC* activity (Fig. 3). The activation of the repressor-

type of ARRs is thought to provide a negative feedback loop in controlling the transient induction of cytokinin early response genes and allows resetting and/or fine-tuning of the physiological state of the cells.

Ectopic expression of the master regulator ARR2 mimics cytokinin responses

In tissue culture, induction of cell proliferation and subsequent shoot formation require cytokinin. To determine whether the same cytokinin signalling pathway is responsible for transcription regulation, cell proliferation, shoot meristem initiation, and leaf formation, we developed a seedling cytokinin response assay. In Figs. 4A and 4B, *Arabidopsis* seedlings were transformed with *Agrobacteria* carrying *GFP, CKI1, CKI1(H405Q), ARR2,* and *ARR6* constructs in the mini-binary vector pCB302 (Xiang et al., *Plant Mol. Biol.* 40: 711-7, 1999). Seedlings transformed with the *Agrobacteria* carrying the binary vector pCB301 without a Bar selection marker were used as a transformation control (vector). The transformed seedlings were selected with glufosinate ammonium (10 μg/ml) and maintained on medium with auxin (IAA) only (Fig. 4A, +IAA, -2IP) or with auxin and cytokinin (Fig. 4B, +IAA, +2IP) for 14 days. The transformed tissues were mostly derived from the shoot apical meristem. The GFP control showed green callus formation with exogenous IAA but promoted shoot formation with exogenous cytokinin. Both CKI1 and ARR2 promoted extensive cell proliferation and shoot formation even without exogenous cytokinin. The experiments were repeated three times with similar results. Compared with the GFP control, the CKI1(H405Q) mutant and ARR6 slightly inhibited cell proliferation on the same medium without or with exogenous cytokinin (Figs. 4A and 4B). The results of this cytokinin seedling assay are consistent with those from protoplast transient expression analyses based on transcription. It is therefore clear that the HK activity of CKI1 is required to perceive and/or initiate cytokinin signalling.

To observe the consequences of constitutive cytokinin signalling *in planta*, the binary vectors expressing CKI1, CKI1(H405Q), ARR2, and ARR6 were

introduced into *Arabidopsis* plants. Ectopic expression of ARR2 in transgenic *Arabidopsis* plants displayed a spectrum of cytokinin-related phenotypes, including reduced apical dominance, increased leaf number and size, altered leaf shape and color, and delayed leaf senescence (Fig. 4C). Transgenic *Arabidopsis* plants carrying the CKI1 construct showed similar phenotypes, but not the CKI1(H405Q) and ARR6 transgenic plants serving as controls. Taken together, CKI1 and ARR2 are positive regulators in the cytokinin signal transduction pathway mediating transcription, cell proliferation, and shoot and leaf formation, as well as delaying leaf senescence. ARR2 could be one of the direct downstream targets of CKI1 and CRE1, and serve as a master regulator in diverse cytokinin responses.

Materials and Methods

The above-mentioned results were obtained using the following materials and methods.

Plasmid Constructs

The 2.4 kb *Arabidopsis ARR6* promoter was fused to the firefly luciferase gene to create the *ARR6-LUC* reporter construct. The *CKI1* and *AHK2* genes were amplified from the *Arabidopsis* genomic DNA by polymerase chain reaction (PCR). The *AHK3, CRE1(CRE1/WOL), AHP1, AHP2, AHP5* and *ARR1,ARR 2, ARR4, ARR5, ARR6, ARR7,* and *ARR10* coding regions were obtained by PCR from an *Arabidopsis* cDNA library (Sheen, *Science* 274: 1900-2, 1996). All of the mutants were generated by QuickChange site-directed mutagenesis (Stratagene). The coding regions of all proteins were tagged with either two copies of the haemagglutinin epitope (DHA) or GFP and inserted into a plant expression vector containing the 35SC4PPDK promoter (Sheen, *supra*) and the nos terminator. All PCR products and the mutations were confirmed by DNA sequencing.

<u>*Arabidopsis* Protoplast Transient Expression Assay</u>

Arabidopsis Bensheim (BE) protoplasts were isolated and transfected as described with some modifications (Kovtun et al., *Proc. Natl. Acad. Sci. U SA* 97: 2940-5 2000). Typically, 2 x 10^4 protoplasts were transfected with 10 μg of plasmid carrying different combinations of a reporter, effectors, and an internal control. For instance, in the experiments shown in Fig. 1A, protoplasts (4 x 10^4) were transfected with 2 μg of *UBQ10-GUS* (internal control) and 20 μg of *ARR6-LUC, GH3-LUC*, or *RD29ALUC* plasmid DNA. Transfected protoplasts were incubated at 1 x 10^4 per ml with/without 1-100 nM of *t*-zeatin for 3-6 hours under the light at 23 °C. The *UBQ10-GUS* construct was used as an internal control to normalize the variations of each transfection in cell numbers, transformation efficiency and cell viability. The results are shown as the means of duplicate samples with the standard deviation. All transient experiments were repeated at least three times with similar results. GFP fluorescence was observed by either Nikon TE200 fluorescent microscopy or Leica TCSNT confocal microscopy.

<u>Protein Immunoprecipitation</u>

Transfected protoplasts were incubated with [^{35}S]-methione (200 μCi/ml) for 6 hours. The effector proteins were immunoprecipitated as described (Sheen, *supra*), analyzed by SDS/PAGE (10 or 12.5 %) and visualized by fluorography.

<u>Seedling Cytokinin Response Assay</u>

Four-day-old *Arabidopsis* BE seedlings were placed on the callus induction medium, containing 0.5 mg/L 2,4-D, 0.05 mg/L BAP, and 0.05 mg/L kinetin, for four days. The seedlings were co-cultivated and transformed with *Agrobacterium tumefaciens* GV3101carrying pCB302 with either *CKI1, CKI1(H405Q), ARR2, ARR6, GFP* under control of the 35SC4PPDK promoter, or the pCB301 binary vector (Xiang et al., *Plant Mol. Biol.* 40:711-7, 1999) alone for three days. Transformed seedlings were then selected on the glufosinate ammonium-containing medium (with either 0.15 mg/L IAA alone or with 0.15

mg/L IAA and 5 mg/L 2IP) and observed up to 4 weeks. The transformed proliferating tissues were mostly derived from the apical shoot meristem of the seedlings.

Transgenic plant analysis

The same constructs analyzed in the *Arabidopsis* protoplast transient expression and seedling assays,including *CKI1*, *CKI1(H405Q)*, *ARR2*, and *ARR6*, were used to generate *Arabidopsis* transgenic plants using the floral dip method and Bar selection as described (Clough and Bent, *Plant J.* 16: 735-43, 1998). More than 200 transgenic plants were obtained with each construct. The *ARR2* and *CKI1* transgenic plants developed branches early, produced more leaves that are bigger and greener but flowered later than the control *CKI1(H405Q)* and *ARR6* transgenic plants. For senescence assay, fully expanded fourth leaves from representative transgenic plants were detached and floated on distilled water in the dark for four days.

Isolation of Sequences Encoding A-type and B-type Response Regulators or Histidine Kinases

The isolation of additional genes encoding A-type and B-type response regulators or histidine kinase genes is accomplished using standard strategies and techniques that are well known in the art.

In one particular example, the B-type response regulator sequence (for example, ARR1, ARR2, or ARR10 sequences), the A-type response regulator sequence, (for example, ARR4, ARR5, ARR6, or ARR7), or the histidine kinase (for example, CRE1 or CKI1), described herein may be used, together with conventional screening methods of nucleic acid hybridization screening, to isolate additional sequences encoding these regulators. Such hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Benton and Davis, *Science* 196: 180, 1977; Grunstein and Hogness, *Proc. Natl. Acad. Sci., USA* 72: 3961, 1975; Ausubel et al. *Current*

Protocols in Molecular Biology, Wiley Interscience, New York; Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York.; and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. In one particular example, all or part of the response regulator gene may be used as a probe to screen a recombinant plant DNA library for genes having sequence identity or similarity to the response regulator gene. Hybridizing sequences are detected by plaque or colony hybridization according to the methods described below.

Alternatively, using all or a portion of the amino acid sequence of a response regulator, one may readily design oligonucleotide probes, including degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any appropriate portion of the response regulator sequence. General methods for designing and preparing such probes are provided, for example, in Ausubel et al., *supra*; and Berger and Kimmel, *supra*. These oligonucleotides are useful for response regulator or histidine kinase sequence isolation, either through their use as probes capable of hybridizing to response regulator complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. (*supra*), or they may be obtained from commercial sources.

As discussed above, response regulator-specific oligonucleotides may also be used as primers in amplification cloning strategies, for example, using PCR. PCR methods are well known in the art and are described, for example, in *PCR Technology*, Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide*

to Methods and Applications, Innis et al., eds., Academic Press, Inc., New York, 1990; and Ausubel et al. (*supra*). Primers are optionally designed to allow cloning of the amplified product into a suitable vector, for example, by including appropriate restriction sites at the 5' and 3' ends of the amplified fragment (as described herein). If desired, response regulator sequences may be isolated using the PCR "RACE" technique, or Rapid Amplification of cDNA Ends (see, e.g., Innis et al. (*supra*)). By this method, oligonucleotide primers based on the response regulator sequence are oriented in the 3' and 5' directions and are used to generate overlapping PCR fragments. These overlapping 3'- and 5'-end RACE products are combined to produce an intact full-length cDNA. This method is described in Innis et al. (*supra*); and Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998, 1988.

Confirmation of a sequence's relatedness to the response regulators may be accomplished by a variety of conventional methods including, but not limited to, sequence comparison of the gene and its expressed product to known response regulators for example those disclosed herein. In addition, the activity of the gene product may be evaluated according to any of the techniques described herein.

Once a response regulator gene is identified, it is cloned according to standard methods and used for the construction of plant expression vectors as described below.

Expression Constructs

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to express the response regulators disclosed herein. For example, an A-type or B-type response regulator may be expressed for the desired effect in any of a number of plant hosts including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, crucifer species, monocots, dicots, or in any plant of commercial or agricultural

significance. Particular examples of suitable plant hosts include, but are not limited to, Conifers, Petunia, Tomato, Potato, Tobacco, *Arabidopsis*, Lettuce, Sunflower, Oilseed rape, Flax, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, *Medicago*, Lotus, *Vigna*, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Grape, Asparagus, Rice, Maize, Millet, Onion, Barley, Orchard grass, Oat, Rye, and Wheat. In addition, as is discussed below, transgenic expression constructs may be expressed in a plant to increase plants yield, shoot formation, leaf growth, photosynthesis, or delay senescence.

Materials for expressing these genes are available from a wide range of sources including the American Type Culture Collection (Rockland, MD); or from any of a number seed companies, for example, W. Atlee Burpee Seed Co. (Warminster, PA), Park Seed Co. (Greenwood, SC), Johnny Seed Co. (Albion, ME), or Northrup King Seeds (Harstville, SC). Descriptions and sources of useful host cells are also found in Vasil, Cell Culture and Somatic Cell Genetics of Plants, Vol I, II, III Laboratory Procedures and Their Applications Academic Press, New York, 1984; Dixon, Plant Cell Culture-A Practical Approach, IRL Press, Oxford University, 1985; Green et al., Plant Tissue and Cell Culture, Academic Press, New York, 1987; and Gasser and Fraley, Science 244: 1293, 1989.

The method of transformation or transfection and the choice of vehicle for expression of the A-type or B-type response regulators, or a histidine kinase will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; Kindle, *Proc. Natl. Acad. Sci., USA* 87: 1228, 1990; Potrykus, *Annu. Rev. Plant Physiol. Plant Mol. Biology* 42: 205, 1991; and BioRad (Hercules, CA) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels et al., 1985, Supp. 1987); Gasser and Fraley (*supra*); Clontech Molecular Biology

Catalog (Catalog 1992/93 Tools for the Molecular Biologist, Palo Alto, CA); and the references cited above. Other expression constructs are described by Fraley et al. (U.S. Pat. No. 5,352,605).

Most preferably, an A-type or B-type response regulator or a histidine kinase is produced by a stably-transfected plant cell line, a transiently-transfected plant cell line, or by a transgenic plant. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Pouwels et al. (*supra*), Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Methods for constructing such cell lines are described in, e.g., Weissbach and Weissbach (*supra*), and Gelvin et al. (*supra*). Typically, plant expression vectors include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, pathogen- or wound-induced, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Once the desired nucleic acid sequence encoding an A-type or B-type response regulators or a histidine kinase is obtained as described above, it may be manipulated in a variety of ways known in the art. For example, where the sequence involves non-coding flanking regions, the flanking regions may be subjected to mutagenesis.

In general, the constructs will involve regulatory regions functional in plants which provide for modified production of the regulator protein or regulator transcript (such as an antisense transcript) as discussed herein. The open reading frame coding for the regulator protein or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the

sequence naturally found in the 5' upstream region of the A-type or B-type response regulator, or a histidine kinase. Numerous other transcription initiation regions are available which provide for constitutive or inducible regulation.

For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5' upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, or leaf development.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the A-type or B-type response regulator or histidine kinase, or any convenient transcription termination region derived from a different gene source. The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived. Plant expression constructs having, for example, A-type or B-type response regulator as the DNA sequence of interest for expression may be employed with a wide variety of plant life. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications as discussed herein. Importantly, this invention is applicable to dicotyledons and monocotyledons, and will be readily applicable to any new or improved transformation or regeneration method.

An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., Nature 313: 810, 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., Plant Cell 2: 591, 1990; Terada and Shimamoto, *Mol. Gen. Genet.* 220: 389, 1990). Moreover, activity of this

promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see .g., Kay et al., *Science* 236: 1299, 1987; Ow et al., *Proc. Natl. Acad. Sci., U.S.A.* 84: 4870, 1987; and Fang et al., *Plant Cell* 1: 141, 1989). In addition, the a minimal 35S promoter may also be used as is described herein.

Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al., *Plant Physiol.* 88: 547, 1988) and the octopine synthase promoter (Fromm et al., *Plant Cell* 1: 977, 1989).

For certain applications, it may be desirable to produce the regulator in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., *Plant Physiol.* 88: 965, 1988; Takahashi and Komeda, *Mol. Gen. Genet.* 219: 365, 1989; and Takahashi et al., *Plant J.* 2: 751, 1992), light-regulated gene expression (e.g., the *Arabidopisis Cab2* photosynthetic, leaf specific promoter described by Mitra et al., *Plant Mol. Biol.* 12: 169-179, 1989; the pea *rbcS-3A* described by Kuhlemeier et al., *Plant Cell* 1: 471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, *Plant Cell* 3: 997, 1991; or the cholorphyll a/b-binding protein gene found in pea described by Simpson et al., *EMBO J.* 4: 2723, 1985), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *Em* gene of wheat described by Marcotte et al., *Plant Cell* 1: 969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and *Arabidopsis* by Straub et al., *Plant Cell* 6: 617, 1994, Shen et al., Plant Cell 7: 295, 1995), wound-induced gene expression (for example, of *wunI* described by Siebertz et al., *Plant Cell* 1: 961, 1989), and organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., *EMBO J.* 6: 1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al.,

EMBO J. 7: 1249, 1988; or the French bean ß-phaseolin gene described by Bustos et al., *Plant Cell* 1: 839, 1989; the vegetative storage protein promoter (soybean vspB) described by Sadka et al. (*Plant Cell* 6: 737-749, 1994)), cycling promoters (e.g., the *Arabidopsis* cdc2a promoter described by Hemerly et al., *Proc. Natl. Acad. Sci. USA* 89: 3295-3299, 1992), senescence-specific promoters (e.g., the *Arabidopsis* SAG12 promoter described by Gan et al, *Science*: 270, 1986-1988, 1995), seed-specific promoters (for example, endosperm-specific or embryo-specific promoters), or pathogen-inducible promoters (for example, PR-1 or β-1,3 glucanase promoters).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., *Genes and Dev.* 1: 1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of the regulator encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 744, 1987; An et al., *Plant Cell* 1: 115, 1989). For example, the 3' terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient

strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the untransformed cells. Some useful concentrations of antibiotics for tobacco transformation include, e.g., 75-100 μg/mL (kanamycin), 20-50 μg/mL (hygromycin), or 5-10 μg/mL (bleomycin). A useful strategy for selection of transformants for herbicide resistance is described, e.g., by Vasil et al., *supra*.

It should be readily apparent to one skilled in the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, e.g., Lichtenstein and Fuller, In: *Genetic Engineering*, Vol 6, Rigby, ed., London, Academic Press, 1987; and Lichtenstein and Draper, In: *DNA Cloning*, Vol II, Glover, ed., Oxford, IRI Press, 1985), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., *Plant Cell* 2: 603,1990); or BioRad Technical Bulletin #1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., *supra*), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., *Plant Cell Physiol.* 23: 451, 1982; or e.g., Zhang and Wu, *Theor. Appl. Genet.* 76: 835, 1988), (5) liposome-mediated DNA uptake (see, .g., Freeman et al., *Plant Cell Physiol.* 25: 1353, 1984), (6)

electroporation protocols (see, e.g., Gelvin et al., *supra*; Dekeyser et al., *supra*; Fromm et al., *Nature* 319: 791, 1986; *Sheen*, Plant Cell 2: 1027, 1990; or Jang and Sheen, *Plant Cell* 6: 1665, 1994), (7) the vortexing method (see, e.g., Kindle, *supra*), and floral dip method (see, e.g., Clough and Bent, *Plant J.* 16, 735-43. (1998)). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer methods are available to transform crops or other host cells, they may be directly applied.

The following is an example outlining one particular technique, an *Agrobacterium*-mediated plant transformation. By this technique, the general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, cloning and DNA modification steps are carried out in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation or electroporation into *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria, for example, streptomycin, and another that will function in plants, for example, a gene encoding kanamycin resistance or herbicide resistance. Also present on the vector are restriction endonuclease sites for the addition of one or more transgenes and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the DNA region that will be transferred to the plant.

In another example, plant cells may be transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic

macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too small for the macroprojectile to pass through. As a result, the plastic macroprojectile smashes against the stopping plate, and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

In general, transfer and expression of transgenes in plant cells are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Whole plants can be regenerated, for example, from single cells, callus tissue, or leaf discs transformed with a plant expression vector according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil, *supra*; Green et al., *supra*; Weissbach and Weissbach, *supra*; and Gelvin et al., *supra*.

In one particular example, a cloned B-type response regulator or histidine kinase construct under the control of the *nos* promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into *Agrobacterium*. Transformation of leaf discs (for example, of tobacco or potato leaf discs), with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (*Science* 227: 1229, 1985). Putative transformants are selected after a few weeks (for example, 3 to 5 weeks) on plant tissue culture media containing kanamycin (e.g. 100 μg/mL). Kanamycin-resistant shoots are then placed on plant tissue culture media without hormones

for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be sowed in a soil-less medium and grown in a greenhouse. Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration of the transgene is accomplished by standard techniques (see, for example, Ausubel et al., *supra*; Gelvin et al., *supra*).

Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can profoundly affect the levels and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization assays using transgene-specific probes (see, e.g., Ausubel et al., *supra*). The RNA-positive plants are then analyzed for protein expression by Western immunoblot analysis using specific antibodies (see, e.g., Ausubel et al., *supra*). In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

Increasing Plant Yield

To test the ability of the genes described herein to improve crop yield or productivity, seeds of transgenic plants expressing a B-type response regulator or histidine kinase are planted in test plots, and their agronomic performance is compared to standard plants using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene. A yield benefit is observed and plants exhibiting the increased yield are advanced for commercialization.

In addition, transgenic plants expressing a B-type response regulator or histidine kinase are field tested for agronomic performance under conditions, including, but not limited to, increased leaf production. When compared to nontransgenic plants, transgenic plants expressing the B-type response regulator exhibit higher yield than their non-transgenic counterparts.

Increasing Plant Shoot Formation

To test the ability of the genes described herein to increase shoot formation, seedlings are transformed with a transgene expressing a B-type response regulator or histidine kinase and shoot formation is measured, for example, by evaluating the development of green callus formation, using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene. Plants exhibiting increased shoot formation relative to control plants are useful in the invention.

Delaying Plant Senescence

To test the ability of the genes described herein to delay senescence, fully expanded fourth leaves of plants expressing a recombinant B-type response regulator or histidine kinase are detached and floated on distilled water in the dark for four days, and their leaf size and quality of green color are compared

using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene.

Enhancing Plant Photosynthesis

To test the ability of the genes and domains described herein to enhance photosynthesis, seedlings are transformed with a transgene expressing a recombinant B-type response regulator or histidine kinase and photosynthesis measured using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene.

Increasing Plant Leaf Growth

To test the ability of the genes described herein to increase leaf growth, seedlings are transformed with a transgene expressing a recombinant B-type response regulator and leaf growth measured, for example, by measuring leaf number and leaf area using techniques familiar to those of skill in the art. Optionally included in this comparison are plants of similar genetic background without the transgene.

Silencing A-type Response Regulator Gene Expression

Plants having decreased expression of an A-type response regulator are useful, for example, for increasing plant productivity, delaying senescence, increasing photosynthesis, increased leaf growth, and increased productivity. Plants having decreased expression of such A-type response regulators are generated according to standard gene silencing methods including, without limitation, co-suppression and antisense methodologies, expression of dominant negative gene products, and creation of plants having mutated genes encoding such regulators.

Co-Suppression

One preferred method of silencing gene expression is co-suppression (also referred to as sense suppression). This technique, which involves introduction of a nucleic acid configured in the sense orientation, has been shown to effectively block the transcription of target genes (see for example, Napoli et al., *Plant Cell*, 2:279-289, 1990 and Jorgensen et al., U.S. Patent No. 5,034,323).

Generally, sense suppression involves transcription of the introduced sequence such as a gene encoding an A-type response regulator. However, co-suppression may also occur where the introduced sequence contains no coding sequence per se, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous gene to be repressed. The introduced sequence generally will be substantially identical to the endogenous gene targeted for repression. Such identity is typically greater than about 50%, but higher identities (for example, 80% or even 95%) are preferred because they result in more effective repression. The effect of co-suppression may also be applied to other proteins within a similar family of genes exhibiting homology or substantial homology. Segments from a gene from one plant can be used directly, for example, to inhibit expression of homologous genes in different plant species.

In sense suppression, the introduced sequence, requiring less than absolute identity, need not be full length, relative to either the primary transcription product or to fully processed mRNA. A higher identity in a shorter than full length sequence compensates for a longer sequence with less identity. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Squences of at least 50 base pairs are preferred, with introduced sequences of greater length being more preferred (see, for example, those methods described by Jorgensen et al., *supra*).

Antisense Suppression

In antisense technology, a nucleic acid segment from the desired plant gene is cloned and operably linked to an expression control region such that the antisense strand of RNA is synthesized. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been shown that antisense RNA inhibits gene expression.

The nucleic acid segment to be introduced in antisense suppression is generally substantially identical to at least a portion of the endogenous gene or genes to be repressed, but need not be identical. The vectors of the present invention therefore can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene. Segments from a gene from one plant can be used, for example, directly to inhibit expression of homologous genes in different plant species.

The introduced sequence also need not be full length relative to either the primary transcription product or to fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Moreover, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. In general, such an antisense sequence will usually be at least 15 base pairs, preferably about 15-200 base pairs, and more preferably 200-2,000 base pairs in length or greater. The antisense sequence may be complementary to all or a portion of the gene to be suppressed and as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. A transcriptional construct expressing a plant A-type response regulator (e.g., those described herein) antisense nucleotide sequence includes, in the direction of transcription, a promoter, the sequence coding for the antisense RNA on the sense strand, and a transcriptional termination region. Antisense sequences may be constructed and expressed as described, for example, in van der Krol et al. (*Gene* 72: 45-50, 1988); Rodermel

et al. (*Cell* 55: 673-681, 1988); Mol et al. (*FEBS Lett.* 268: 427-430, 1990); Weigel and Nilsson (*Nature* 377: 495-500, 1995); Cheung et al., (*Cell* 82: 383-393, 1995); and Shewmaker et al. (U.S. Patent No. 5,107,065).

In one working example, antisense expression of the gene encoding A-type response regulators of *Arabidopsis* or an A-type response regulator homolog is used to increase plant yield. In one particular approach, a plant expression vector is constructed that contains the cDNA sequence of A-type response regulator homolog in antisense orientation that is constitutively expressed under the control of the rice actin promoter described by Wu et al. (WO 91/09948). This expression vector is then used to transform rice plants according to conventional methods, for example, using the methods described in Hiei et al. (*Plant J.* 6:271-282, 1994).

Dominant Negatives

Transgenic plants expressing a transgene encoding a dominant negative gene product of an A-type response regulator are assayed in artificial environments or in the field to demonstrate that the transgene increases yield, delays senescence, increases photosynthesis, or increases leaf formation on the plant expressing the gene. Dominant negative transgenes are constructed according to methods known in the art. Typically, a dominant negative gene encodes a mutant A-type response regulator which, when overexpressed, disrupts the activity of the wild type polypeptide.

Mutants

Plants having decreased expression of an A-type response regulator are also generated using standard mutagenesis methodologies. Such mutagenesis methods include, without limitation, treatment of seeds with ethyl methylsulfate, ethylmethylsulfonate (EMS), or fast neutron irradiation, as well as T-DNA insertion methodologies. Expression of an A-type response regulator and increased yield phenotypes in mutated and non-mutated lines are evaluated according to standard procedures (for example, those described herein). Mutated

plants having decreased expression of a gene encoding a A-type response regulator exhibit increased yield relative to their non-mutated counterparts.

Use

The invention described herein is useful for a variety of agricultural and commercial purposes including, but not limited to, improving and enhancing photosynthesis, promoting cell proliferation, shoot meristem formation, promoting leaf development, increasing crop yields, improving crop and ornamental quality, and reducing agricultural production costs. In particular, ectopic expression of a B-type response regulator (such as ARR2 or an ARR2 homolog) in a plant cell provides a method for increasing plant productivity, photosynthesis, and delaying senescence. The invention is especially useful for crop plants such as tomato, potato, cotton, pepper, maize, wheat, rice, and legumes such as soybean.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

CLAIMS

1. A method for increasing yield in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a B-type response regulator operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein said B-type response regulator is expressed in the cells of said transgenic plant, thereby increasing yield in said plant.

2. The method of claim 1, wherein said B-type response regulator is a crucifer B-type response regulator.

3. The method of claim 2, wherein said crucifer B-type response regulator is selected from the group consisting of ARR1, ARR2, and ARR10.

4. The method of claim 1, wherein said DNA encoding said B-type response regulator is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

5. A method for increasing yield in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein expression of said transgene reduces expression of an A-type response regulator in the cells of said plant, thereby increasing yield in said plant.

6. The method of claim 5, wherein said A-type response regulator is a crucifer A-type response regulator.

7. The method of claim 6, wherein said crucifer A-type response regulator is selected from the group consisting of ARR4, ARR5, ARR6, and ARR7.

8. The method of claim 5, wherein said transgene expresses antisense A-type response regulator RNA.

9. The method of claim 5, wherein said transgene expresses a dominant negative A-type response regulator.

10. The method of claim 5, wherein said transgene co-suppresses expression of A-type response regulator.

11. The method of claim 5, wherein said DNA encoding said transgene is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

12. A method for increasing shoot formation in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a B-type response regulator operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein said B-type response regulator is expressed in the cells of said plant, thereby increasing shoot formation in said plant.

13. The method of claim 12, wherein said B-type response regulator is a crucifer B-type response regulator.

14. The method of claim 13, wherein said crucifer B-type response regulator is selected from the group consisting of ARR1, ARR2, and ARR10.

15. The method of claim 12, wherein said DNA encoding said B-type response regulator is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

16. A method for increasing shoot formation in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein expression of said transgene reduces expression of an A-type response regulator in the cells of said plant, thereby increasing shoot formation in said plant.

17. The method of claim 16, wherein said A-type response regulator is a crucifer A-type response regulator.

18. The method of claim 17, wherein said crucifer A-type response regulator is selected from the group consisting of ARR4, ARR5, ARR6, and ARR7.

19. The method of claim 16, wherein said transgene expresses antisense A-type response regulator RNA.

20. The method of claim 16, wherein said transgene expresses a dominant negative A-type response regulator.

21. The method of claim 16, wherein said transgene co-suppresses expression of A-type response regulator.

22. The method of claim 16, wherein said transgene is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

23. A method for delaying senescence in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a B-type response regulator operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein said B-type response regulator is expressed in the cells of said plant, thereby delaying senesence in said plant.

24. The method of claim 23, wherein said B-type response regulator is a crucifer B-type response regulator.

25. The method of claim 24, wherein said crucifer B-type response regulator is selected from the group consisting of ARR1, ARR2, and ARR10.

26. The method of claim 23, wherein said DNA encoding said B-type response regulator is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

27. A method for delaying senescence in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein expression of said transgene reduces expression of an A-type response regulator in the cells of said plant, thereby delaying senescence in said plant.

28. The method of claim 27, wherein said A-type response regulator is a crucifer A-type response regulator.

29. The method of claim 28, wherein said crucifer A-type response regulator is selected from the group consisting of ARR4, ARR5, ARR6, and ARR7.

30. The method of claim 27, wherein said transgene expresses antisense A-type response regulator RNA.

31. The method of claim 27, wherein said transgene expresses a dominant negative A-type response regulator.

32. The method of claim 27, wherein said transgene co-suppresses expression of A-type response regulator.

33. The method of claim 27, wherein said transgene is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

34. A method for increasing yield in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein histidine kinase is expressed in the cells of said plant, thereby increasing yield in said plant.

35. The method of claim 34, wherein said histidine kinase is a crucifer histidine kinase.

36. The method of claim 35, wherein said crucifer histidine kinase is CKI1 or CRE1.

37. The method of claim 34, wherein said DNA encoding said histidine kinase is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

38. A method for increasing shoot formation in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein histidine kinase is expressed in the cells of said plant, thereby increasing shoot formation in said plant.

39. The method of claim 38, wherein said histidine kinase is a crucifer histidine kinase.

40. The method of claim 39, wherein said crucifer histidine kinase is CKI1 or CRE1.

41. The method of claim 38, wherein said DNA encoding said histidine kinase is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

42. A method for delaying senescence in a plant, said method comprising the steps of:

(a) introducing into plant cells a transgene comprising DNA encoding a histidine kinase operably linked to a promoter functional in plant cells to yield transformed plant cells; and

(b) regenerating a plant from said transformed cells, wherein histidine kinase is expressed in the cells of said plant, thereby delaying senescence in said plant.

43. The method of claim 42, wherein said histidine kinase is a crucifer histidine kinase.

44. The method of claim 43, wherein said crucifer histidine kinase is CKI1 or CRE1.

45. The method of claim 42, wherein said DNA encoding said histidine kinase is constitutively expressed, inducibly expressed, expressed in a cell-specific, tissue-specific, or organ-specific manner, or expressed under cycling conditions.

46. A plant or plant component comprising at least one transgene encoding (i) an A-type response regulator polypeptide, (ii) an antisense A-type response regulator RNA, (iii) a dominant negative A-type response regulator polypeptide, (iv) a B-type response regulator polypeptide, (v) an antisense B-type response regulator RNA, (vi) a dominant negative B-type response regulator, (vii) an antisense HK RNA, (viii) a dominant negative HK polypeptide, or any combination of (i) –(viii).

47. The plant or plant component of claim 48, further comprising a transgene encoding a histidine kinase polypeptide.

48. A plant or plant component comprising:

(a) a first transgene encoding (i) an A-type response regulator polypeptide, (ii) an antisense A-type response regulator RNA, or (iii) a dominant negative A-type response regulator polypeptide or combination thereof;

(b) a second transgene encoding (iv) a B-type response regulator polypeptide, (v) an antisense B-type response regulator RNA, (vi) a dominant negative B-type response regulator or combination thereof, and

(c) a third transgene encoding (vii) a HK polypeptide; (viii) an antisense HK RNA, (ix) a dominant negative HK polypeptide or combination thereof.

49. The plant or plant component of claims 46, 47, or 48, wherein said plant or plant component is selected from the group consisting of wheat, rice, maize, barley, potato, soybean, tomato, oats, cotton, and sunflower.

FIG. 1A

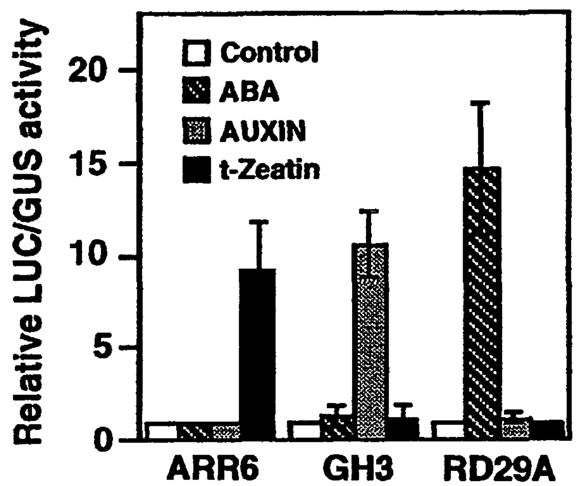

Relative LUC/GUS activity
0
5
10
15
20
Control
ABA
AUXIN
t-Zeatin
ARR6
GH3
RD29A

FIG. 1B

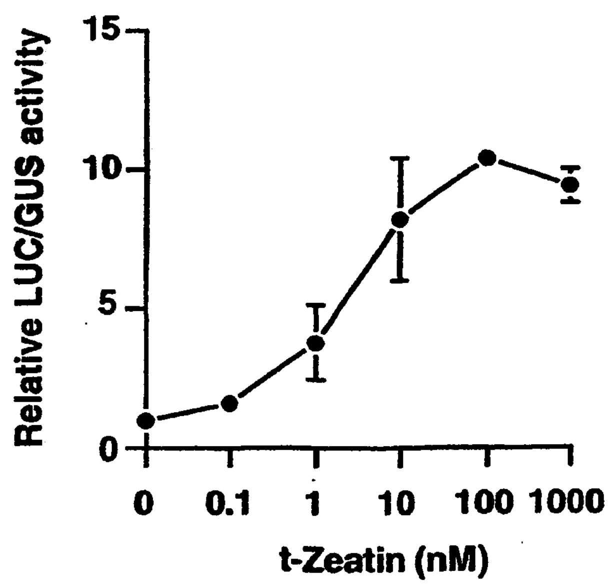

Relative LUC/GUS activity
0
5
10
15
0
0.1
1
10
100
1000
t-Zeatin (nM)

FIG. 1C

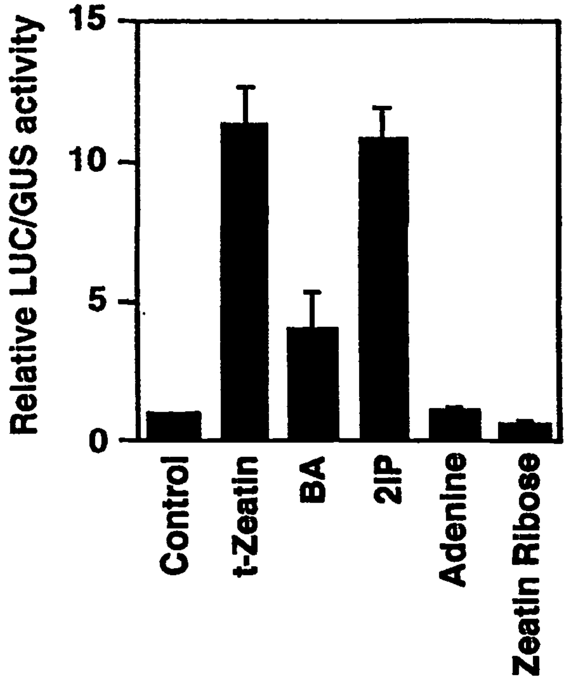

Relative LUC/GUS activity
15
10
5
0
Control
t-Zeatin
BA
2IP
Adenine
Zeatin Ribose

FIG. 1D

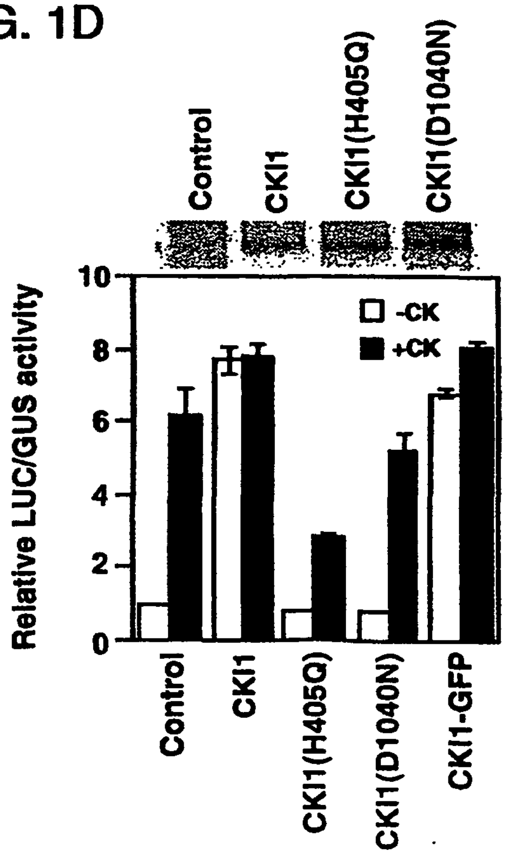
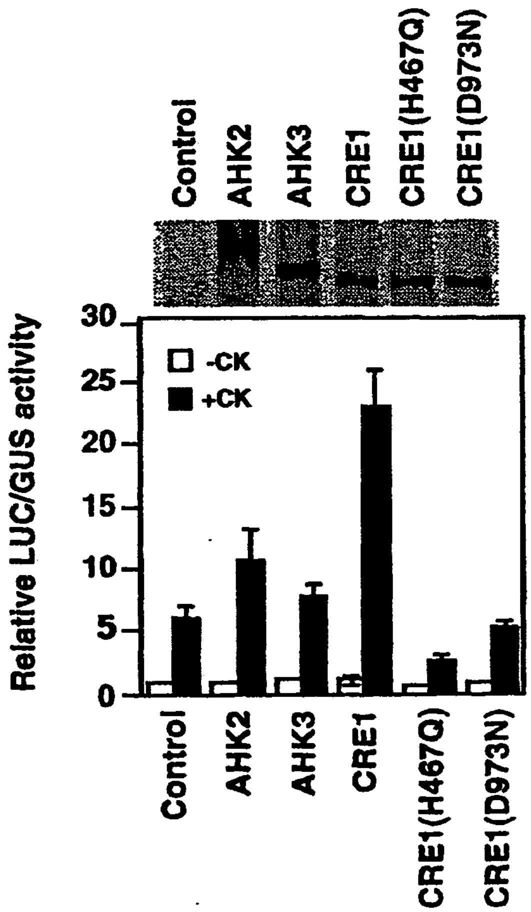

Control
CKI1
CKI1(H405Q)
CKI1(D1040N)
Relative LUC/GUS activity
10
8
6
4
2
0
-CK
+CK
Control
CKI1
CKI1(H405Q)
CKI1(D1040N)
CKI1-GFP

FIG. 1E


Control
AHK2
AHK3
CRE1
CRE1(H467Q)
CRE1(D973N)
Relative LUC/GUS activity
30
25
20
15
10
5
0
-CK
+CK
Control
AHK2
AHK3
CRE1
CRE1(H467Q)
CRE1(D973N)

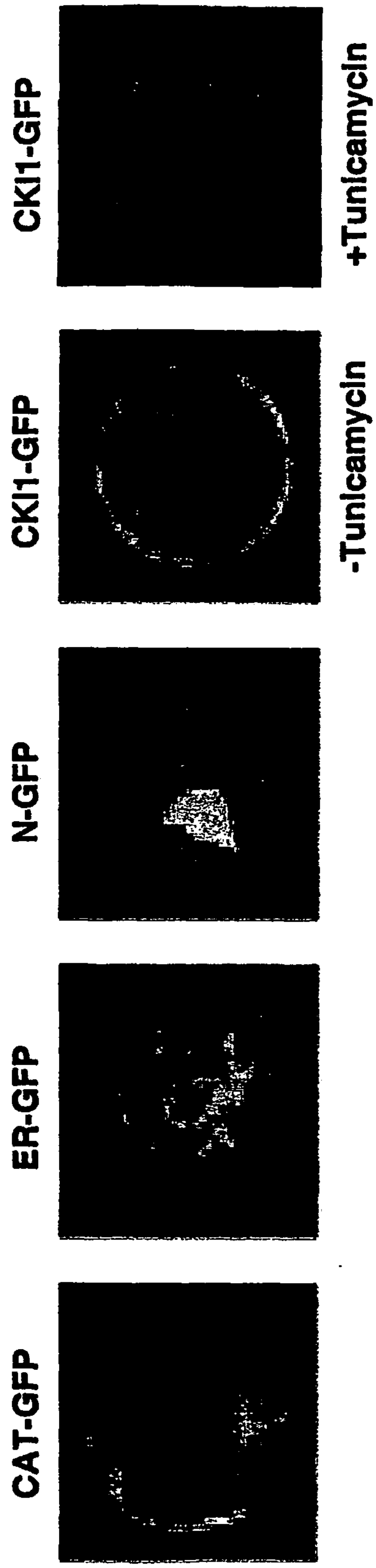

CKI1-GFP
+Tunicamycin
CKI1-GFP
-Tunicamycin
N-GFP
ER-GFP
CAT-GFP

FIG. 1F

FIG. 2A

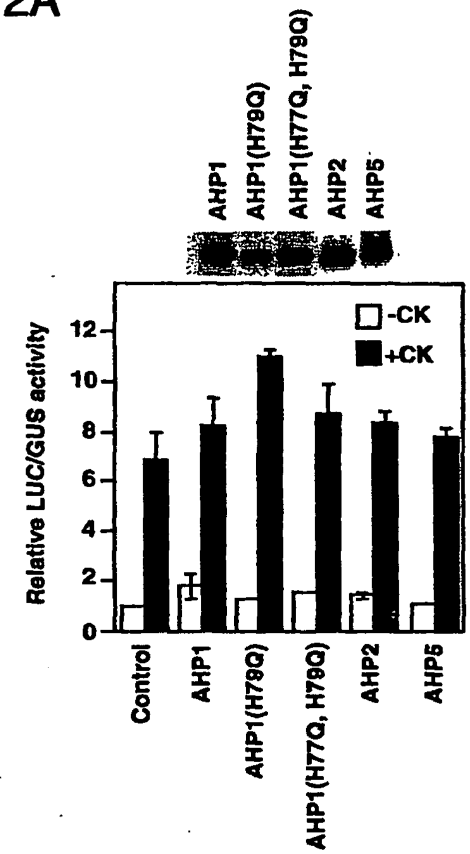

AHP1
AHP1(H79Q)
AHP1(H77Q, H79Q)
AHP2
AHP5
-CK
+CK
Relative LUC/GUS activity
0
2
4
6
8
10
12
Control
AHP1
AHP1(H79Q)
AHP1(H77Q, H79Q)
AHP2
AHP5

FIG. 2B

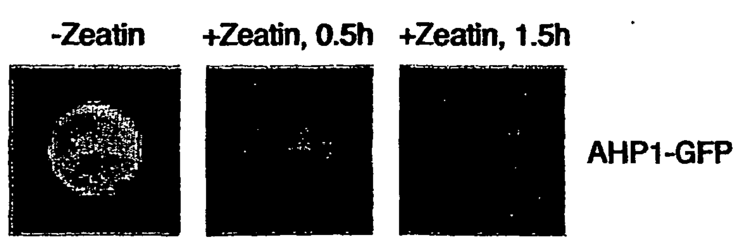

-Zeatin
+Zeatin, 0.5h
+Zeatin, 1.5h
AHP1-GFP

FIG. 3A

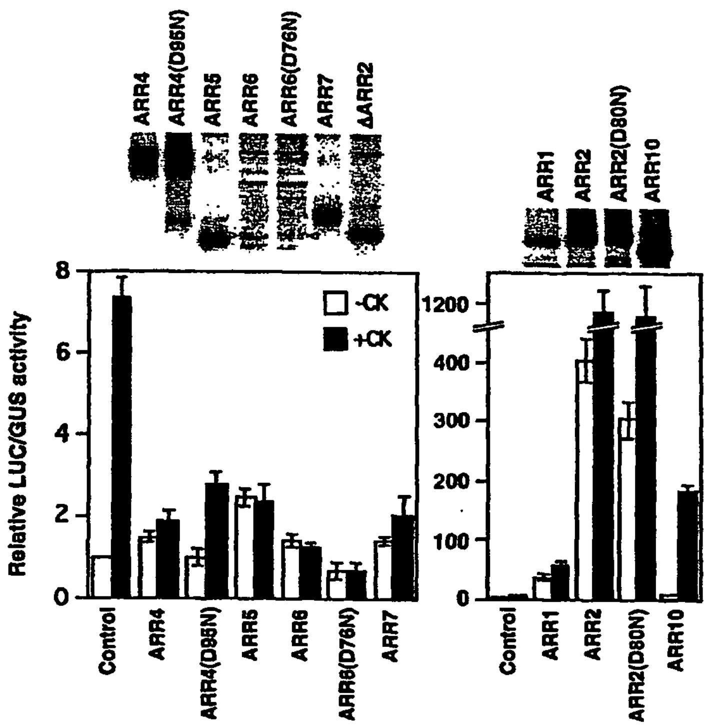
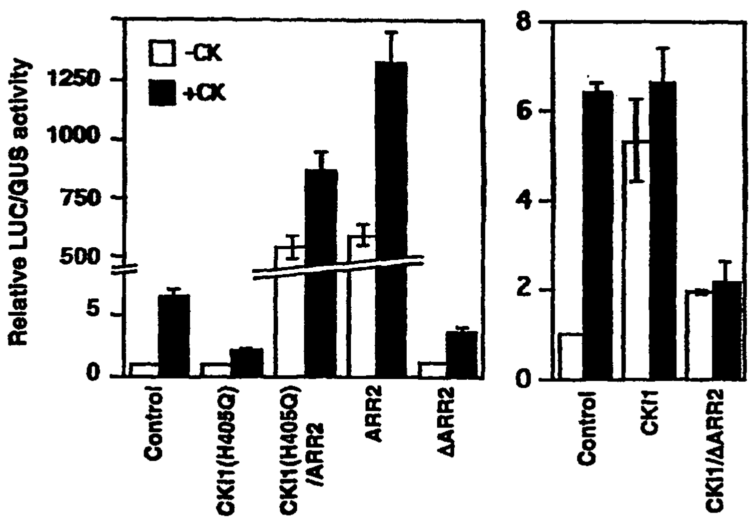

ARR4
ARR4(D95N)
ARR5
ARR6
ARR6(D76N)
ARR7
ΔARR2
ARR1
ARR2
ARR2(D80N)
ARR10
Relative LUC/GUS activity
-CK
+CK
Control
ARR4
ARR4(D95N)
ARR5
ARR6
ARR6(D76N)
ARR7
Control
ARR1
ARR2
ARR2(D80N)
ARR10

FIG. 3B


Relative LUC/GUS activity
-CK
+CK
Control
CKI1(H405Q)
CKI1(H405Q)/ARR2
ARR2
ΔARR2
Control
CKI1
CKI1/ΔARR2

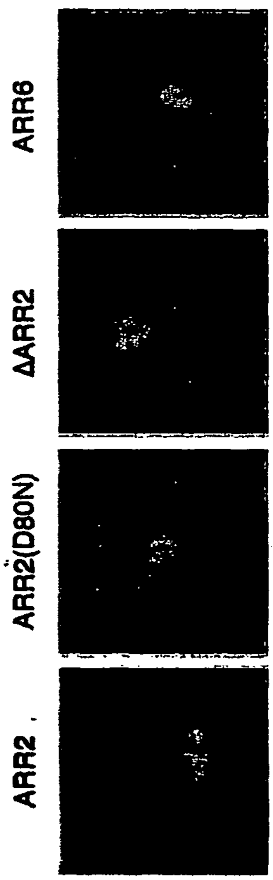
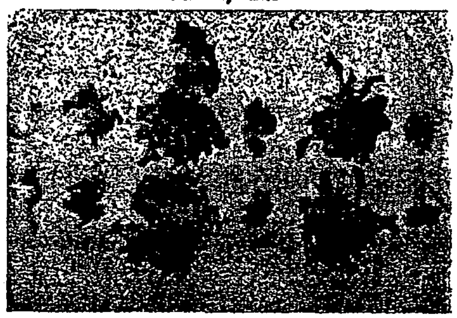


FIG. 3C

FIG. 4A

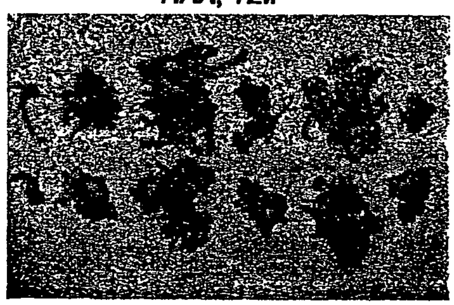
+IAA, -2IP



Vector GFP CKI1 CKI1(H405Q) ARR2 ARR6

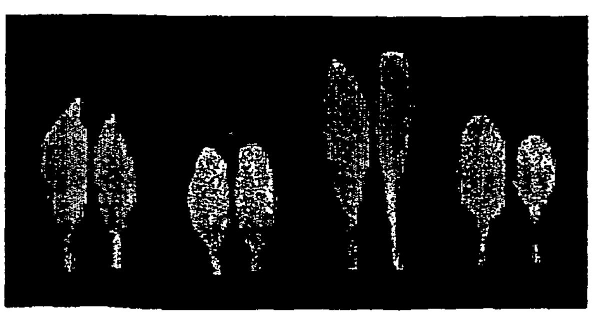
FIG. 4B

+IAA, +2IP



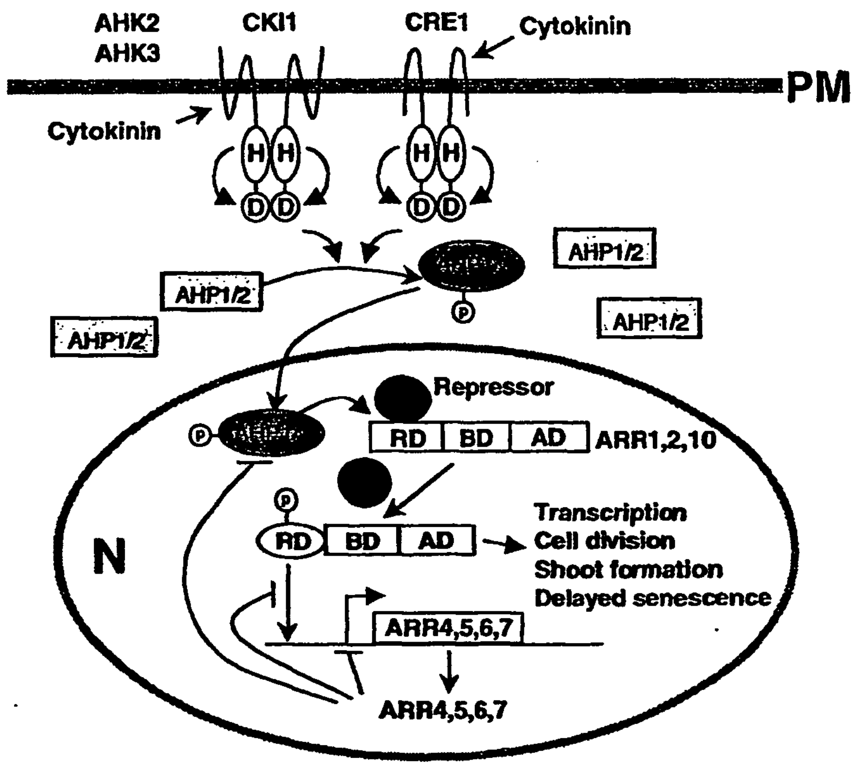
Vector GFP CKI1 CKI1(H405Q) ARR2 ARR6

FIG. 4C



CKI1 CKI1(H405Q) ARR2 ARR6

FIG. 5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PM
Cytokinin
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D
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D
D
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AHP1/2
AHP1/2
AHP1/2
P
Repressor
P
RD
BD
AD
ARR1,2,10
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RD
BD
AD
Transcription
Cell division
Shoot formation
Delayed senescence
N
ARR4,5,6,7
ARR4,5,6,7

SEQUENCE LISTING

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tcattcaagc tggtggtata gttcctcaag tggatattaa ctaaagagac tagtccataa 600
gaagaaaaaa gatgatgact ttctttcttt agtttctctt ctaaattatt ttggatttgg 660
tgtttgctca aaaactcaat aaaatatgtg caaaaagaaa caaaaacaag tgatggttgt 720
ttataaatca gtagtatgta ttgtttgatc tcatccgaga aaattgaaac cattggacta 780
atgaatgtga tgataatata tattggtttg cttctcc 817

<210> 23
<211> 510
<212> DNA
<213> Arabidopsis thaliana

<400> 23
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cgtttctctt tatcaacagg gatttctgga taatcagttc tcagagttga gaaagttgca 120
agatgaagga acccctgatt ttgtagctga agttgtctct ctattcttcg acgactgttc 180
caagcttatt aataccatgt ctatatccct ggagcggcca gataatgtgg atttcaaaca 240
ggtggattca ggtgttcatc aactcaaggg tagtagctcc agtgtcggtg caaggagggt 300
gaaaaatgtg tgcatatctt tcaaggaatg ttgcgatgtt cagaaccgtg aagggtgtct 360
aaggtgttta cagcaggtgg attatgaata taagatgtta aagactaaac ttcaggatct 420
ctttaattta gagaaacaga tcctccaagc tggaggtaca attcctcaag tggatataaa 480
ttagaccgat gcgtttctcg attatgcaaa 510

<210> 24
<211> 465
<212> DNA
<213> Artificial Sequence

<220>
<223> derived from Arabidopsis thaliana

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gggatttgg acagccagtt cttgcagctg caacaactac aagatgaaag caatccagat 120
tttgtttcac aagttgtcac actcttcttc caagactctg ataggattct caatgatctc 180

tcactttccc tagatcaaca agttgtagac tttaaaaaag ttgatcccca tgttcaacaa 240
ctcaaaggta gcagctccag tataggagca cagagagtta agaatgcttg tgttgtcttc 300
cgcagcttct gcgagcagca aaatgtcgaa gcatgtcata gatgtttgca acaagtgaag 360
caagagtatt atcttgtgaa aaacagatta gagactctgt tcaagctgga gcaacagatt 420
gtagcttctg gtggaatgat cccggccgtc gaactcggat tttga 465

<210> 25
<211> 465
<212> DNA
<213> Artificial Sequence

<220>
<223> derived from Arabidopsis thaliana

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tttgtttcac aagttgtcac actcttcttc caagactctg ataggattct caatgatctc 180
tcactttccc tagatcaaca agttgtagac tttaaaaaag ttgatcccca agttcaacaa 240
ctcaaaggta gcagctccag tataggagca cagagagtta agaatgcttg tgttgtcttc 300
cgcagcttct gcgagcagca aaatgtcgaa gcatgtcata gatgtttgca acaagtgaag 360
caagagtatt atcttgtgaa aaacagatta gagactctgt tcaagctgga gcaacagatt 420
gtagcttctg gtggaatgat cccggccgtc gaactcggat tttga 465

<210> 26
<211> 1176
<212> PRT
<213> Arabidopsis thaliana

<400> 26
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35 40 45
Leu Gly Arg Ser Gln Lys Glu Glu Thr Asp Ser Cys Asn Gly Glu Glu
50 55 60
Lys Val Leu Tyr Arg His Gln Asn Val Thr Arg Ser Glu Ile His Asp
65 70 75 80
Leu Val Ser Leu Phe Ser Asp Ser Asp Gln Val Thr Ser Phe Glu Cys
85 90 95
His Lys Glu Ser Ser Pro Gly Met Trp Thr Asn Tyr Gly Ile Thr Cys
100 105 110
Ser Leu Ser Val Arg Ser Asp Lys Gln Glu Thr Arg Gly Leu Pro Trp
115 120 125
Asn Leu Gly Leu Gly His Ser Ile Ser Ser Thr Ser Cys Met Cys Gly
130 135 140
Asn Leu Glu Pro Ile Leu Gln Gln Pro Glu Asn Leu Glu Glu Glu Asn
145 150 155 160
His Glu Glu Gly Leu Glu Gln Gly Leu Ser Ser Tyr Leu Arg Asn Ala
165 170 175
Trp Trp Cys Leu Ile Leu Gly Val Leu Val Cys His Lys Ile Tyr Val
180 185 190
Ser His Ser Lys Ala Arg Gly Glu Arg Lys Glu Lys Val His Leu Gln
195 200 205
Glu Ala Leu Ala Pro Lys Lys Gln Gln Gln Arg Ala Gln Thr Ser Ser
210 215 220

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Arg Gly Ala Gly Arg Trp Arg Lys Asn Ile Leu Leu Leu Gly Ile Leu
225                 230                 235                 240
Gly Gly Val Ser Phe Ser Val Trp Trp Phe Trp Asp Thr Asn Glu Glu
                245                 250                 255
Ile Ile Met Lys Arg Arg Glu Thr Leu Ala Asn Met Cys Asp Glu Arg
            260                 265                 270
Ala Arg Val Leu Gln Asp Gln Phe Asn Val Ser Leu Asn His Val His
        275                 280                 285
Ala Leu Ser Ile Leu Val Ser Thr Phe His His Gly Lys Ile Pro Ser
    290                 295                 300
Ala Ile Asp Gln Arg Thr Phe Glu Glu Tyr Thr Glu Arg Thr Asn Phe
305                 310                 315                 320
Glu Arg Pro Leu Thr Ser Gly Val Ala Tyr Ala Leu Lys Val Pro His
                325                 330                 335
Ser Glu Arg Glu Lys Phe Glu Lys Glu His Gly Trp Ala Ile Lys Lys
            340                 345                 350
Met Glu Thr Glu Asp Gln Thr Val Val Gln Asp Cys Val Pro Glu Asn
        355                 360                 365
Phe Asp Pro Ala Pro Ile Gln Asp Glu Tyr Ala Pro Val Ile Phe Ala
    370                 375                 380
Gln Glu Thr Val Ser His Ile Val Ser Val Asp Met Met Ser Gly Glu
385                 390                 395                 400
Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Ala Ser Gly Lys Gly Val
                405                 410                 415
Leu Thr Ser Pro Phe Lys Leu Leu Lys Ser Asn His Leu Gly Val Val
            420                 425                 430
Leu Thr Phe Ala Val Tyr Asp Thr Ser Leu Pro Pro Asp Ala Thr Glu
        435                 440                 445
Glu Gln Arg Val Glu Ala Thr Ile Gly Tyr Leu Gly Ala Ser Tyr Asp
    450                 455                 460
Met Pro Ser Leu Val Glu Lys Leu Leu His Gln Leu Ala Ser Lys Gln
465                 470                 475                 480
Thr Ile Ala Val Asp Val Tyr Asp Thr Thr Asn Thr Ser Gly Leu Ile
                485                 490                 495
Lys Met Tyr Gly Ser Glu Ile Gly Asp Ile Ser Glu Gln His Ile Ser
            500                 505                 510
Ser Leu Asp Phe Gly Asp Pro Ser Arg Asn His Glu Met His Cys Arg
        515                 520                 525
Phe Lys His Lys Leu Pro Ile Pro Trp Thr Ala Ile Thr Pro Ser Ile
    530                 535                 540
Leu Val Leu Val Ile Thr Phe Leu Val Gly Tyr Ile Leu Tyr Glu Ala
545                 550                 555                 560
Ile Asn Arg Ile Ala Thr Val Glu Glu Asp Cys Gln Lys Met Arg Glu
                565                 570                 575
Leu Lys Ala Arg Ala Glu Ala Ala Asp Ile Ala Lys Ser Gln Phe Leu
            580                 585                 590
Ala Thr Val Ser His Glu Ile Arg Thr Pro Met Asn Gly Val Leu Gly
        595                 600                 605
Met Leu Lys Met Leu Met Asp Thr Asp Leu Asp Ala Lys Gln Met Asp
    610                 615                 620
Tyr Ala Gln Thr Ala His Gly Ser Gly Lys Asp Leu Thr Ser Leu Ile
625                 630                 635                 640
Asn Glu Val Leu Asp Gln Ala Lys Ile Glu Ser Gly Arg Leu Glu Leu
                645                 650                 655
Glu Asn Val Pro Phe Asp Met Arg Phe Ile Leu Asp Asn Val Ser Ser
            660                 665                 670
Leu Leu Ser Gly Lys Ala Asn Glu Lys Gly Ile Glu Leu Ala Val Tyr
        675                 680                 685
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Val Ser Ser Gln Val Pro Asp Val Val Val Gly Asp Pro Ser Arg Phe
690 695 700
Arg Gln Ile Ile Thr Asn Leu Val Gly Asn Ser Ile Lys Phe Thr Gln
705 710 715 720
Glu Arg Gly His Ile Phe Ile Ser Val His Leu Ala Asp Glu Val Lys
725 730 735
Glu Pro Leu Thr Ile Glu Asp Ala Val Leu Lys Gln Arg Leu Ala Leu
740 745 750
Gly Cys Ser Glu Ser Gly Glu Thr Val Ser Gly Phe Pro Ala Val Asn
755 760 765
Ala Trp Gly Ser Trp Lys Asn Phe Lys Thr Cys Tyr Ser Thr Glu Ser
770 775 780
Gln Asn Ser Asp Gln Ile Lys Leu Leu Val Thr Val Glu Asp Thr Gly
785 790 795 800
Val Gly Ile Pro Val Asp Ala Gln Gly Arg Ile Phe Thr Pro Phe Met
805 810 815
Gln Ala Asp Ser Ser Thr Ser Arg Thr Tyr Gly Gly Thr Gly Ile Gly
820 825 830
Leu Ser Ile Ser Lys Arg Leu Val Glu Leu Met Gln Gly Glu Met Gly
835 840 845
Phe Val Ser Glu Pro Gly Ile Gly Ser Thr Phe Ser Phe Thr Gly Val
850 855 860
Phe Gly Lys Ala Glu Thr Asn Thr Ser Ile Thr Lys Leu Glu Arg Phe
865 870 875 880
Asp Leu Ala Ile Gln Glu Phe Thr Gly Leu Arg Ala Leu Val Ile Asp
885 890 895
Asn Arg Asn Ile Arg Ala Glu Val Thr Arg Tyr Glu Leu Arg Arg Leu
900 905 910
Gly Ile Ser Ala Asp Ile Val Ser Ser Leu Arg Met Ala Cys Thr Cys
915 920 925
Cys Ile Ser Lys Leu Glu Asn Leu Ala Met Ile Leu Ile Asp Lys Asp
930 935 940
Ala Trp Asn Lys Glu Glu Phe Ser Val Leu Asp Glu Leu Phe Thr Arg
945 950 955 960
Ser Lys Val Thr Phe Thr Arg Val Pro Lys Ile Phe Leu Leu Ala Thr
965 970 975
Ser Ala Thr Leu Thr Glu Arg Ser Glu Met Lys Ser Thr Gly Leu Ile
980 985 990
Asp Glu Val Val Ile Lys Pro Leu Arg Met Ser Val Leu Ile Cys Cys
995 1000 1005
Leu Gln Glu Thr Leu Val Asn Gly Lys Lys Arg Gln Pro Asn Arg Gln
1010 1015 1020
Arg Arg Asn Leu Gly His Leu Leu Arg Glu Lys Gln Ile Leu Val Val
1025 1030 1035 1040
Asp Asp Asn Leu Val Asn Arg Arg Val Ala Glu Gly Ala Leu Lys Lys
1045 1050 1055
Tyr Gly Ala Ile Val Thr Cys Val Glu Ser Gly Lys Ala Ala Leu Ala
1060 1065 1070
Met Leu Lys Pro Pro His Asn Phe Asp Ala Cys Phe Met Asp Leu Gln
1075 1080 1085
Met Pro Glu Met Asp Gly Phe Glu Ala Thr Arg Arg Val Arg Glu Leu
1090 1095 1100
Glu Arg Glu Ile Asn Lys Lys Ile Ala Ser Gly Glu Val Ser Ala Glu
1105 1110 1115 1120
Met Phe Cys Lys Phe Ser Ser Trp His Val Pro Ile Leu Ala Met Thr
1125 1130 1135
Ala Asp Val Ile Gln Ala Thr His Glu Glu Cys Met Lys Cys Gly Met
1140 1145 1150

Asp Gly Tyr Val Ser Lys Pro Phe Glu Glu Glu Val Leu Tyr Thr Ala
1155 1160 1165
Val Ala Arg Phe Phe Glu Pro Cys
1170 1175

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<211> 1036
<212> PRT
<213> Arabidopsis thaliana

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Ile Glu Asp Lys Ser Gly Leu Leu Val Gly Ser Val Gly Asp Leu Glu
35 40 45
Lys Thr Lys Met Thr Thr Leu Lys Lys Lys Asn Lys Met Trp Phe Trp
50 55 60
Asn Lys Ile Ser Ser Ser Gly Leu Lys Ile Pro Ser Phe Ser Tyr Gln
65 70 75 80
Phe Leu Gly Ser Val Lys Phe Asn Lys Ala Trp Trp Arg Lys Leu Val
85 90 95
Val Val Trp Val Val Phe Trp Val Leu Val Ser Ile Trp Thr Phe Trp
100 105 110
Tyr Phe Ser Ser Gln Ala Met Glu Lys Arg Lys Glu Thr Leu Ala Ser
115 120 125
Met Cys Asp Glu Arg Ala Arg Met Leu Gln Asp Gln Phe Asn Val Ser
130 135 140
Met Asn His Val Gln Ala Met Ser Ile Leu Ile Ser Thr Phe His His
145 150 155 160
Gly Lys Ile Pro Ser Ala Ile Asp Gln Arg Thr Phe Ser Glu Tyr Thr
165 170 175
Asp Arg Thr Ser Phe Glu Arg Pro Leu Thr Ser Gly Val Ala Tyr Ala
180 185 190
Met Arg Val Leu His Ser Glu Arg Glu Glu Phe Glu Arg Gln Gln Gly
195 200 205
Trp Thr Ile Arg Lys Met Tyr Ser Leu Glu Gln Asn Pro Val His Lys
210 215 220
Asp Asp Tyr Asp Leu Glu Ala Leu Glu Pro Ser Pro Val Gln Glu Glu
225 230 235 240
Tyr Ala Pro Val Ile Phe Ala Gln Asp Thr Val Ser His Val Val Ser
245 250 255
Leu Asp Met Leu Ser Gly Lys Glu Asp Arg Glu Asn Val Leu Arg Ala
260 265 270
Arg Ser Ser Gly Lys Gly Val Leu Thr Ala Pro Phe Pro Leu Ile Lys
275 280 285
Thr Asn Arg Leu Gly Val Ile Leu Thr Phe Ala Val Tyr Lys Arg Asp
290 295 300
Leu Pro Ser Asn Ala Thr Pro Lys Glu Arg Ile Glu Ala Thr Asn Gly
305 310 315 320
Tyr Leu Gly Gly Val Phe Asp Ile Glu Ser Leu Val Glu Asn Leu Leu
325 330 335
Gln Gln Leu Ala Ser Lys Gln Thr Ile Leu Val Asn Val Tyr Asp Ile
340 345 350
Thr Asn His Ser Gln Pro Ile Ser Met Tyr Gly Thr Asn Val Ser Ala
355 360 365

Asp Gly Leu Glu Arg Val Ser Pro Leu Ile Phe Gly Asp Pro Leu Arg
370 375 380
Lys His Glu Met Arg Cys Arg Phe Lys Gln Lys Pro Pro Trp Pro Val
385 390 395 400
Leu Ser Met Val Thr Ser Phe Gly Ile Leu Val Ile Ala Leu Leu Val
405 410 415
Ala His Ile Ile His Ala Thr Val Ser Arg Ile His Lys Val Glu Glu
420 425 430
Asp Cys Asp Lys Met Lys Gln Leu Lys Lys Lys Ala Glu Ala Ala Asp
435 440 445
Val Ala Lys Ser Gln Phe Leu Ala Thr Val Ser His Glu Ile Arg Thr
450 455 460
Pro Met Asn Gly Val Leu Gly Met Leu His Met Leu Met Asp Thr Glu
465 470 475 480
Leu Asp Val Thr Gln Gln Asp Tyr Val Arg Thr Ala Gln Ala Ser Gly
485 490 495
Lys Ala Leu Val Ser Leu Ile Asn Glu Val Leu Asp Gln Ala Lys Ile
500 505 510
Glu Ser Gly Lys Leu Glu Leu Glu Glu Val Arg Phe Asp Leu Arg Gly
515 520 525
Ile Leu Asp Asp Val Leu Ser Leu Phe Ser Ser Lys Ser Gln Gln Lys
530 535 540
Gly Val Glu Leu Ala Val Tyr Ile Ser Asp Arg Val Pro Asp Met Leu
545 550 555 560
Ile Gly Asp Pro Gly Arg Phe Arg Gln Ile Leu Thr Asn Leu Met Gly
565 570 575
Asn Ser Ile Lys Phe Thr Glu Lys Gly His Ile Phe Val Thr Val His
580 585 590
Leu Val Asp Glu Leu Phe Glu Ser Ile Asp Gly Glu Thr Ala Ser Ser
595 600 605
Pro Glu Ser Thr Leu Ser Gly Leu Pro Val Ala Asp Arg Gln Arg Ser
610 615 620
Trp Glu Asn Phe Lys Ala Phe Ser Ser Asn Gly His Arg Ser Phe Glu
625 630 635 640
Pro Ser Pro Pro Asp Ile Asn Leu Ile Val Ser Val Glu Asp Thr Gly
645 650 655
Val Gly Ile Pro Val Glu Ala Gln Ser Arg Ile Phe Thr Pro Phe Met
660 665 670
Gln Val Gly Pro Ser Ile Ser Arg Thr His Gly Gly Thr Gly Ile Gly
675 680 685
Leu Ser Ile Ser Lys Cys Leu Val Gly Leu Met Lys Gly Glu Ile Gly
690 695 700
Phe Ser Ser Thr Pro Lys Val Gly Ser Thr Phe Thr Phe Thr Ala Val
705 710 715 720
Phe Ser Asn Gly Met Gln Pro Ala Glu Arg Lys Asn Asp Asn Asn Gln
725 730 735
Pro Ile Phe Ser Glu Phe Arg Gly Met Lys Ala Val Val Val Asp His
740 745 750
Arg Pro Ala Arg Ala Lys Val Ser Trp Tyr His Phe Gln Arg Leu Gly
755 760 765
Ile Arg Val Glu Val Val Pro Arg Val Glu Gln Ala Leu His Tyr Leu
770 775 780
Lys Ile Gly Thr Thr Thr Val Asn Met Ile Leu Ile Glu Gln Glu Ile
785 790 795 800
Trp Asn Arg Glu Ala Asp Asp Phe Ile Lys Lys Leu Gln Lys Asp Pro
805 810 815
Leu Phe Leu Ser Pro Lys Leu Ile Leu Leu Ala Asn Ser Val Glu Ser
820 825 830

Ser Ile Ser Glu Ala Leu Cys Thr Gly Ile Asp Pro Pro Ile Val Ile
835 840 845
Val Lys Pro Leu Arg Ala Ser Met Leu Ala Ala Thr Leu Gln Arg Gly
850 855 860
Leu Gly Ile Gly Ile Arg Glu Pro Pro Gln His Lys Gly Pro Pro Ala
865 870 875 880
Leu Ile Leu Arg Asn Leu Leu Leu Gly Arg Lys Ile Leu Ile Val Asp
885 890 895
Asp Asn Asn Val Asn Leu Arg Val Ala Ala Gly Ala Leu Lys Lys Tyr
900 905 910
Gly Ala Asp Val Val Cys Ala Glu Ser Gly Ile Lys Ala Ile Ser Leu
915 920 925
Leu Lys Pro Pro His Glu Phe Asp Ala Cys Phe Met Asp Ile Gln Met
930 935 940
Pro Glu Met Asp Gly Phe Glu Ala Thr Arg Arg Ile Arg Asp Met Glu
945 950 955 960
Glu Glu Met Asn Lys Arg Ile Lys Asn Gly Glu Ala Leu Ile Val Glu
965 970 975
Asn Gly Asn Lys Thr Ser Trp His Leu Pro Val Leu Ala Met Thr Ala
980 985 990
Asp Val Ile Gln Ala Thr His Glu Glu Cys Leu Lys Cys Gly Met Asp
995 1000 1005
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<211> 1057
<212> PRT
<213> Arabidopsis thaliana

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35 40 45
Pro Arg Lys Ile Asp Phe Trp Arg Ser Gly Leu Met Gly Phe Ala Lys
50 55 60
Met Gln Gln Gln Gln Gln Leu Gln His Ser Val Ala Val Lys Met Asn
65 70 75 80
Asn Asn Asn Asn Asn Asp Leu Met Gly Asn Lys Lys Gly Ser Thr Phe
85 90 95
Ile Gln Glu His Arg Ala Leu Leu Pro Lys Ala Leu Ile Leu Trp Ile
100 105 110
Ile Ile Val Gly Phe Ile Ser Ser Gly Ile Tyr Gln Trp Met Asp Asp
115 120 125
Ala Asn Lys Ile Arg Arg Glu Glu Val Leu Val Ser Met Cys Asp Gln
130 135 140
Arg Ala Arg Met Leu Gln Asp Gln Phe Ser Val Ser Val Asn His Val
145 150 155 160
His Ala Leu Ala Ile Leu Val Ser Thr Phe His Tyr His Lys Asn Pro
165 170 175
Ser Ala Ile Asp Gln Glu Thr Phe Ala Glu Tyr Thr Ala Arg Thr Ala
180 185 190

Phe Glu Arg Pro Leu Leu Ser Gly Val Ala Tyr Ala Glu Lys Val Val
        195   200   205
Asn Phe Glu Arg Glu Met Phe Glu Arg Gln His Asn Trp Val Ile Lys
    210   215   220
Thr Met Asp Arg Gly Glu Pro Ser Pro Val Arg Asp Glu Tyr Ala Pro
225   230   235   240
Val Ile Phe Ser Gln Asp Ser Val Ser Tyr Leu Glu Ser Leu Asp Met
                245   250   255
Met Ser Gly Glu Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Glu Thr
            260   265   270
Gly Lys Ala Val Leu Thr Ser Pro Phe Arg Leu Leu Glu Thr His His
        275   280   285
Leu Gly Val Val Leu Thr Phe Pro Val Tyr Lys Ser Ser Leu Pro Glu
    290   295   300
Asn Pro Thr Val Glu Glu Arg Ile Ala Ala Thr Ala Gly Tyr Leu Gly
305   310   315   320
Gly Ala Phe Asp Val Glu Ser Leu Val Glu Asn Leu Leu Gly Gln Leu
                325   330   335
Ala Gly Asn Gln Ala Ile Val Val His Val Tyr Asp Ile Thr Asn Ala
            340   345   350
Ser Asp Pro Leu Val Met Tyr Gly Asn Gln Asp Glu Glu Ala Asp Arg
        355   360   365
Ser Leu Ser His Glu Ser Lys Leu Asp Phe Gly Asp Pro Phe Arg Lys
    370   375   380
His Lys Met Ile Cys Arg Tyr His Gln Lys Ala Pro Ile Pro Leu Asn
385   390   395   400
Val Leu Thr Thr Val Pro Leu Phe Phe Ala Ile Gly Phe Leu Val Gly
                405   410   415
Tyr Ile Leu Tyr Gly Ala Ala Met His Ile Val Lys Val Glu Asp Asp
            420   425   430
Phe His Glu Met Gln Glu Leu Lys Val Arg Ala Glu Ala Ala Asp Val
        435   440   445
Ala Lys Ser Gln Phe Leu Ala Thr Val Ser His Glu Ile Arg Thr Pro
    450   455   460
Met Asn Gly Ile Leu Gly Met Leu Ala Met Leu Leu Asp Thr Glu Leu
465   470   475   480
Ser Ser Thr Gln Arg Asp Tyr Ala Gln Thr Ala Gln Val Cys Gly Lys
                485   490   495
Ala Leu Ile Ala Leu Ile Asn Glu Val Leu Asp Arg Ala Lys Ile Glu
            500   505   510
Ala Gly Lys Leu Glu Leu Glu Ser Val Pro Phe Asp Ile Arg Ser Ile
        515   520   525
Leu Asp Asp Val Leu Ser Leu Phe Ser Glu Glu Ser Arg Asn Lys Ser
    530   535   540
Ile Glu Leu Ala Val Phe Val Ser Asp Lys Val Pro Glu Ile Val Lys
545   550   555   560
Gly Asp Ser Gly Arg Phe Arg Gln Ile Ile Ile Asn Leu Val Gly Asn
                565   570   575
Ser Val Lys Phe Thr Glu Lys Gly His Ile Phe Val Lys Val His Leu
            580   585   590
Ala Glu Gln Ser Lys Asp Glu Ser Glu Pro Lys Asn Ala Leu Asn Gly
        595   600   605
Gly Val Ser Glu Glu Met Ile Val Val Ser Lys Gln Ser Ser Tyr Asn
    610   615   620
Thr Leu Ser Gly Tyr Glu Ala Ala Asp Gly Arg Asn Ser Trp Asp Ser
625   630   635   640
Phe Lys His Leu Val Ser Glu Glu Gln Ser Leu Ser Glu Phe Asp Ile
                645   650   655

Ser Ser Asn Val Arg Leu Met Val Ser Ile Glu Asp Thr Gly Ile Gly
660 665 670
Ile Pro Leu Val Ala Gln Gly Arg Val Phe Met Pro Phe Met Gln Ala
675 680 685
Asp Ser Ser Thr Ser Arg Asn Tyr Gly Gly Thr Gly Ile Gly Leu Ser
690 695 700
Ile Ser Lys Cys Leu Val Glu Leu Met Arg Gly Gln Ile Asn Phe Ile
705 710 715 720
Ser Arg Pro His Ile Gly Ser Thr Phe Trp Phe Thr Ala Val Leu Glu
725 730 735
Lys Cys Asp Lys Cys Ser Ala Ile Asn His Met Lys Lys Pro Asn Val
740 745 750
Glu His Leu Pro Ser Thr Phe Lys Gly Met Lys Ala Ile Val Val Asp
755 760 765
Ala Lys Pro Val Arg Ala Ala Val Thr Arg Tyr His Met Lys Arg Leu
770 775 780
Gly Ile Asn Val Asp Val Val Thr Ser Leu Lys Thr Ala Val Val Ala
785 790 795 800
Ala Ala Ala Phe Glu Arg Asn Gly Ser Pro Leu Pro Thr Lys Pro Gln
805 810 815
Leu Asp Met Ile Leu Val Glu Lys Asp Ser Trp Ile Ser Thr Glu Asp
820 825 830
Asn Asp Ser Glu Ile Arg Leu Leu Asn Ser Arg Thr Asn Gly Asn Val
835 840 845
His His Lys Ser Pro Lys Leu Ala Leu Phe Ala Thr Asn Ile Thr Asn
850 855 860
Ser Glu Phe Asp Arg Ala Lys Ser Ala Gly Phe Ala Asp Thr Val Ile
865 870 875 880
Met Lys Pro Leu Arg Ala Ser Met Ile Gly Ala Cys Leu Gln Gln Val
885 890 895
Leu Glu Leu Arg Lys Thr Arg Gln Gln His Pro Glu Gly Ser Ser Pro
900 905 910
Ala Thr Leu Lys Ser Leu Leu Thr Gly Lys Lys Ile Leu Val Val Asp
915 920 925
Asp Asn Ile Val Asn Arg Arg Val Ala Ala Gly Ala Leu Lys Lys Phe
930 935 940
Gly Ala Glu Val Val Cys Ala Glu Ser Gly Gln Val Ala Leu Gly Leu
945 950 955 960
Leu Gln Ile Pro His Thr Phe Asp Ala Cys Phe Met Asp Ile Gln Met
965 970 975
Pro Gln Met Asp Gly Phe Glu Ala Thr Arg Gln Ile Arg Met Met Glu
980 985 990
Lys Glu Thr Lys Glu Lys Thr Asn Leu Glu Trp His Leu Pro Ile Leu
995 1000 1005
Ala Met Thr Ala Asp Val Ile His Ala Thr Tyr Glu Glu Cys Leu Lys
1010 1015 1020
Ser Gly Met Asp Gly Tyr Val Ser Lys Pro Phe Glu Glu Glu Asn Leu
1025 1030 1035 1040
Tyr Lys Ser Val Ala Lys Ser Phe Lys Pro Asn Pro Ile Ser Pro Ser
1045 1050 1055
Ser

<210> 29
<211> 1057
<212> PRT
<213> Artificial Sequence

<220>
<223> derived from Arabidopsis thaliana

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20 25 30
Asp Phe Tyr Gln Leu Gly Gly Gly Gly Ala Leu Asn Ser Ser Glu Lys
35 40 45
Pro Arg Lys Ile Asp Phe Trp Arg Ser Gly Leu Met Gly Phe Ala Lys
50 55 60
Met Gln Gln Gln Gln Gln Leu Gln His Ser Val Ala Val Lys Met Asn
65 70 75 80
Asn Asn Asn Asn Asn Asp Leu Met Gly Asn Lys Lys Gly Ser Thr Phe
85 90 95
Ile Gln Glu His Arg Ala Leu Leu Pro Lys Ala Leu Ile Leu Trp Ile
100 105 110
Ile Ile Val Gly Phe Ile Ser Ser Gly Ile Tyr Gln Trp Met Asp Asp
115 120 125
Ala Asn Lys Ile Arg Arg Glu Glu Val Leu Val Ser Met Cys Asp Gln
130 135 140
Arg Ala Arg Met Leu Gln Asp Gln Phe Ser Val Ser Val Asn His Val
145 150 155 160
His Ala Leu Ala Ile Leu Val Ser Thr Phe His Tyr His Lys Asn Pro
165 170 175
Ser Ala Ile Asp Gln Glu Thr Phe Ala Glu Tyr Thr Ala Arg Thr Ala
180 185 190
Phe Glu Arg Pro Leu Leu Ser Gly Val Ala Tyr Ala Glu Lys Val Val
195 200 205
Asn Phe Glu Arg Glu Met Phe Glu Arg Gln His Asn Trp Val Ile Lys
210 215 220
Thr Met Asp Arg Gly Glu Pro Ser Pro Val Arg Asp Glu Tyr Ala Pro
225 230 235 240
Val Ile Phe Ser Gln Asp Ser Val Ser Tyr Leu Glu Ser Leu Asp Met
245 250 255
Met Ser Gly Glu Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Glu Thr
260 265 270
Gly Lys Ala Val Leu Thr Ser Pro Phe Arg Leu Leu Glu Thr His His
275 280 285
Leu Gly Val Val Leu Thr Phe Pro Val Tyr Lys Ser Ser Leu Pro Glu
290 295 300
Asn Pro Thr Val Glu Glu Arg Ile Ala Ala Thr Ala Gly Tyr Leu Gly
305 310 315 320
Gly Ala Phe Asp Val Glu Ser Leu Val Glu Asn Leu Leu Gly Gln Leu
325 330 335
Ala Gly Asn Gln Ala Ile Val Val His Val Tyr Asp Ile Thr Asn Ala
340 345 350
Ser Asp Pro Leu Val Met Tyr Gly Asn Gln Asp Glu Glu Ala Asp Arg
355 360 365
Ser Leu Ser His Glu Ser Lys Leu Asp Phe Gly Asp Pro Phe Arg Lys
370 375 380
His Lys Met Ile Cys Arg Tyr His Gln Lys Ala Pro Ile Pro Leu Asn
385 390 395 400
Val Leu Thr Thr Val Pro Leu Phe Phe Ala Ile Gly Phe Leu Val Gly
405 410 415
Tyr Ile Leu Tyr Gly Ala Ala Met His Ile Val Lys Val Glu Asp Asp
420 425 430

Phe His Glu Met Gln Glu Leu Lys Val Arg Ala Glu Ala Ala Asp Val
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Ala Lys Ser Gln Phe Leu Ala Thr Val Ser Gln Glu Ile Arg Thr Pro
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Met Asn Gly Ile Leu Gly Met Leu Ala Met Leu Leu Asp Thr Glu Leu
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Ser Ser Thr Gln Arg Asp Tyr Ala Gln Thr Ala Gln Val Cys Gly Lys
485 490 495
Ala Leu Ile Ala Leu Ile Asn Glu Val Leu Asp Arg Ala Lys Ile Glu
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Ala Gly Lys Leu Glu Leu Glu Ser Val Pro Phe Asp Ile Arg Ser Ile
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Gly Asp Ser Gly Arg Phe Arg Gln Ile Ile Ile Asn Leu Val Gly Asn
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Ser Val Lys Phe Thr Glu Lys Gly His Ile Phe Val Lys Val His Leu
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Ala Glu Gln Ser Lys Asp Glu Ser Glu Pro Lys Asn Ala Leu Asn Gly
595 600 605
Gly Val Ser Glu Glu Met Ile Val Val Ser Lys Gln Ser Ser Tyr Asn
610 615 620
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625 630 635 640
Phe Lys His Leu Val Ser Glu Glu Gln Ser Leu Ser Glu Phe Asp Ile
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Ser Ser Asn Val Arg Leu Met Val Ser Ile Glu Asp Thr Gly Ile Gly
660 665 670
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Asp Ser Ser Thr Ser Arg Asn Tyr Gly Gly Thr Gly Ile Gly Leu Ser
690 695 700
Ile Ser Lys Cys Leu Val Glu Leu Met Arg Gly Gln Ile Asn Phe Ile
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Ser Arg Pro His Ile Gly Ser Thr Phe Trp Phe Thr Ala Val Leu Glu
725 730 735
Lys Cys Asp Lys Cys Ser Ala Ile Asn His Met Lys Lys Pro Asn Val
740 745 750
Glu His Leu Pro Ser Thr Phe Lys Gly Met Lys Ala Ile Val Val Asp
755 760 765
Ala Lys Pro Val Arg Ala Ala Val Thr Arg Tyr His Met Lys Arg Leu
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Gly Ile Asn Val Asp Val Val Thr Ser Leu Lys Thr Ala Val Val Ala
785 790 795 800
Ala Ala Ala Phe Glu Arg Asn Gly Ser Pro Leu Pro Thr Lys Pro Gln
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Leu Asp Met Ile Leu Val Glu Lys Asp Ser Trp Ile Ser Thr Glu Asp
820 825 830
Asn Asp Ser Glu Ile Arg Leu Leu Asn Ser Arg Thr Asn Gly Asn Val
835 840 845
His His Lys Ser Pro Lys Leu Ala Leu Phe Ala Thr Asn Ile Thr Asn
850 855 860
Ser Glu Phe Asp Arg Ala Lys Ser Ala Gly Phe Ala Asp Thr Val Ile
865 870 875 880
Met Lys Pro Leu Arg Ala Ser Met Ile Gly Ala Cys Leu Gln Gln Val
885 890 895

Leu Glu Leu Arg Lys Thr Arg Gln Gln His Pro Glu Gly Ser S r Pro
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Ala Thr Leu Lys Ser Leu Leu Thr Gly Lys Lys Ile Leu Val Val Asp
915 920 925
Asp Asn Ile Val Asn Arg Arg Val Ala Ala Gly Ala Leu Lys Lys Phe
930 935 940
Gly Ala Glu Val Val Cys Ala Glu Ser Gly Gln Val Ala Leu Gly Leu
945 950 955 960
Leu Gln Ile Pro His Thr Phe Asp Ala Cys Phe Met Asp Ile Gln Met
965 970 975
Pro Gln Met Asp Gly Phe Glu Ala Thr Arg Gln Ile Arg Met Met Glu
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Lys Glu Thr Lys Glu Lys Thr Asn Leu Glu Trp His Leu Pro Ile Leu
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Ala Met Thr Ala Asp Val Ile His Ala Thr Tyr Glu Glu Cys Leu Lys
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Ser Gly Met Asp Gly Tyr Val Ser Lys Pro Phe Glu Glu Glu Asn Leu
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35 40 45
Pro Arg Lys Ile Asp Phe Trp Arg Ser Gly Leu Met Gly Phe Ala Lys
50 55 60
Met Gln Gln Gln Gln Gln Leu Gln His Ser Val Ala Val Lys Met Asn
65 70 75 80
Asn Asn Asn Asn Asn Asp Leu Met Gly Asn Lys Lys Gly Ser Thr Phe
85 90 95
Ile Gln Glu His Arg Ala Leu Leu Pro Lys Ala Leu Ile Leu Trp Ile
100 105 110
Ile Ile Val Gly Phe Ile Ser Ser Gly Ile Tyr Gln Trp Met Asp Asp
115 120 125
Ala Asn Lys Ile Arg Arg Glu Glu Val Leu Val Ser Met Cys Asp Gln
130 135 140
Arg Ala Arg Met Leu Gln Asp Gln Phe Ser Val Ser Val Asn His Val
145 150 155 160
His Ala Leu Ala Ile Leu Val Ser Thr Phe His Tyr His Lys Asn Pro
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Ser Ala Ile Asp Gln Glu Thr Phe Ala Glu Tyr Thr Ala Arg Thr Ala
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Phe Glu Arg Pro Leu Leu Ser Gly Val Ala Tyr Ala Glu Lys Val Val

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Thr Met Asp Arg Gly Glu Pro Ser Pro Val Arg Asp Glu Tyr Ala Pro
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Met Ser Gly Glu Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Glu Thr
            260                 265                 270
Gly Lys Ala Val Leu Thr Ser Pro Phe Arg Leu Leu Glu Thr His His
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Leu Gly Val Val Leu Thr Phe Pro Val Tyr Lys Ser Ser Leu Pro Glu
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Asn Pro Thr Val Glu Glu Arg Ile Ala Ala Thr Ala Gly Tyr Leu Gly
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Val Leu Thr Thr Val Pro Leu Phe Phe Ala Ile Gly Phe Leu Val Gly
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Tyr Ile Leu Tyr Gly Ala Ala Met His Ile Val Lys Val Glu Asp Asp
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Phe His Glu Met Gln Glu Leu Lys Val Arg Ala Glu Ala Ala Asp Val
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Ser Val Lys Phe Thr Glu Lys Gly His Ile Phe Val Lys Val His Leu
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Gly Val Ser Glu Glu Met Ile Val Val Ser Lys Gln Ser Ser Tyr Asn
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Thr Leu Ser Gly Tyr Glu Ala Ala Asp Gly Arg Asn Ser Trp Asp Ser
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Ser Ser Asn Val Arg Leu Met Val Ser Ile Glu Asp Thr Gly Ile Gly
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Ile Pro Leu Val Ala Gln Gly Arg Val Phe Met Pro Phe Met Gln Ala
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Asp Ser Ser Thr Ser Arg Asn Tyr Gly Gly Thr Gly Ile Gly Leu Ser
690 695 700
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740 745 750
Glu His Leu Pro Ser Thr Phe Lys Gly Met Lys Ala Ile Val Val Asp
755 760 765
Ala Lys Pro Val Arg Ala Ala Val Thr Arg Tyr His Met Lys Arg Leu
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Gly Ile Asn Val Asp Val Val Thr Ser Leu Lys Thr Ala Val Val Ala
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His His Lys Ser Pro Lys Leu Ala Leu Phe Ala Thr Asn Ile Thr Asn
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885 890 895
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Leu Gln Ile Pro His Thr Phe Asp Ala Cys Phe Met Asn Ile Gln Met
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Pro Gln Met Asp Gly Phe Glu Ala Thr Arg Gln Ile Arg Met Met Glu
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Tyr Lys Ser Val Ala Lys Ser Phe Lys Pro Asn Pro Ile Ser Pro Ser
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Ser